CHEMOATTRACTANT G PROTEIN-COUPLED RECEPTORS IN HUMAN MAST CELLS

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ABSTRACT

Mast cells are crucial effector cells in host defense and immunoregulation. They are equipped with an impressive array of receptor systems which allows them to rapidly respond to pathogen-associated signals. G protein-coupled receptors (GPCRs) mediate mast cell activation and mediator release in response to various stimuli. The effects are differential; while some GPCR can induce chemotaxis, adhesion, degranulation and mediator release, others can stimulate chemotaxis and adhesion but not degranulation. Signaling through GPCRs can also modulate antigen-mediated activation of mast cells. The effect mediated via a GPCR depends on the G protein it couples with. Signal transduction via $G\alpha_i$ initiates cell activation, whereas $G\alpha_s$ downregulates cell activation. My thesis investigated the roles of novel stimulatory and inhibitory GPCRs in human mast cells. I hypothesized that human mast cells express both stimulatory and inhibitory chemoattractant GPCRs which differentially activate mast cells but chemotaxis pathway itself remains conserved. For the first sub-hypothesis, expression and function of FPRL1 receptor in human mast cells was investigated. I showed that Laboratory of Allergic Diseases 2 (LAD2) human mast cells expressed cell surface FPRL1. Stimulation of LAD2 cells with a novel α-cationic antimicrobial peptide, pleurocidin, induced mast cell chemotaxis, adhesion, degranulation and production of lipid mediators, cytokines, and chemokines. The effect was sensitive to pertussis toxin, indicating that FPRL1 mediates stimulatory signaling in human mast cells via $G\alpha_i$ protein.

In the next sub-hypothesis, expression and function of C5a receptors, C5aR and C5L2, in human mast cells was investigated. While C5aR is a known $G\alpha_i$-coupled GPCR in human mast cells, the signaling mechanism for C5L2 is unknown. My data showed that LAD2 human mast cells express cell surface C5L2 but not C5aR. C5a activated LAD2 cells to migrate, adhere and produce cytokines and chemokines. The effects were

abolished in mast cells in which C5L2 expression was knocked-down using lentiviral C5L2 shRNA. Moreover, the effect of C5a on cell adhesion was pertussis toxin-dependent, indicating that C5L2 mediates stimulatory signaling in human mast cells via $G\alpha_i$ proteins.

In the final sub-hypothesis, function of C3a receptor, C3aR, in human mast cells was investigated in conjugation with adenosine receptor signaling. Stimulatory signal transduction through $G\alpha_i$-coupled C3aR in human mast cells is well-established. To test the expression and function of inhibitory GPCRs, adenosine receptors were utilized. I showed that while adenosine did not activate human mast cells by itself, it inhibited C3a-mediated activation of human mast cell adhesion, chemotaxis, degranulation, and chemokine production. An A_{2A} receptor specific agonist, CGS 21680, blocked the inhibitory effect of adenosine, indicating that $G\alpha_s$-coupled adenosine receptor exert inhibitory effects on $G\alpha_i$-coupled GPCR activation in human mast cells.

Overall, my thesis research provides crucial information on the effects of novel chemoattractant GPCRs in human mast cells. While these receptors differentially modulated various human mast cell functions either positively via $G\alpha_i$-coupled GPCRs or negatively via $G\alpha_s$-coupled GPCRs, chemotaxis remained conserved and occurred via a $G\alpha_i$-dependent signal transduction mechanism.

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DEDICATION

To my family,

with all my love and gratitude

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A1	Adenosine Receptor 1
A2A	Adenosine Receptor 2a
A2B	Adenosine Receptor 2b
A3	Adenosine Receptor 3
AC	Adenylyl cyclase
aIgE	Anti-IgE
AM	Acetoxymethyl
AP-2	Adaptor protein complex-2
AR	Adenosine receptor
ASM	Airway smooth muscle
ASP	Acylation stimulating protein, C3a desArg
ATP	Adenosine triphosphate
BALF	Bronchoaveolar lavage fluid
bla	Beta-lactamase
BMMC	Bone marrow-derived mast cells
BSA	Bovine serum albumin
C3a	Complement component 3a
C3aR	C3a receptor
C4a	Complement component C4a
c48/80	Compound 48/80
C5a	Complement component 5a
C5aR	C5a receptor
C5L2	C5a receptor-like 2
C5L2kd	C5L2 knockdown
Ca^{2+}	Calcium
cADPR	Cyclic adenosine diphosphate-ribose

cAMP	Cyclic adenosine monophosphate
CAP	Cationic antimicrobial peptide
CBA	Cytometric bead array
CBMC	Cord blood-derived mast cells
CCL	Chemokine (C-C motif) ligand
cGMP	Cyclic guanosine monophosphate
c-Kit	Stem cell factor receptor gene
CREB	cAMP-response element binding protein
CTMC	Connective tissue mast cells
CXCL	Chemokine (C-X-C motif) ligand
CysLT	Cysteinyl leukotriene
DAG	Diacylglycerol
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
ECP	Eosinophil cationic protein
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EP1-4	Prostaglandin receptor 1-4
ERK	Extracellular signal regulated kinase
ET-1	Endothelin-1
FBS	Fetal bovine serum
FcεRI	High affinity receptor for IgE
FcγRI	High affinity receptor for IgG
FcγRII	Low affinity receptor for IgG
FcγRIII	A second low affinity receptor for IgG
FGF2	Fibroblast growth factor 2
Fmlf	N-formyl-methionyl-leucyl-phenylalanine
FPR	N-formyl peptide receptor

FPRL1	N-formyl peptide receptor-like 1
FPRL2	N-formyl peptide receptor-like 2
FRET	Fluorescence resonance energy transfer
Gα	GDP nucleotide-binding alpha-subunit
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GIRK	G protein-regulated inward rectifier potassium channel
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
GDP	Guanosine diphosphate
GPI	Glycosylphosphatidylinositol
GRK	GPCR kinase
GTP	Guanosine triphosphate
h	Hour
H_1	Histamine receptor 1
H_2	Histamine receptor 2
HEK(293)	Human embryonic kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HuMC	Human peripheral blood $CD34^+$ cell-derived mast cells
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMC	Intestinal mast cells
IP_3	Inositol-1,4,5-triphosphate
IP-10	Inducible protein-10
ITAM	Immunoreceptor tyrosine-based activation motifs
JNK	c-Jun N-terminal kinase

K_d	Dissociation constant
kDa	kilodalton
Kit	Stem cell factor receptor, CD117
LAD	Laboratory of Allergic Diseases
LMC	Lung mast cells
LPS	Lipopolysaccharide
LT	Leukotriene
LXA_4	Lipoxin A_4
MAPK	Mitogen-activated protein kinase
MC/9	Murine mast cell line
MCP	Mast cell protease
MCP-1	Monocyte chemoattractant protein-1
MC_T	$Tryptase^+$ mast cells
MC_{TC}	$Tryptase^+$ $Chymase^+$ mast cells
MFI	Mean fluorescent intensity
MIG	Monokine induced by interferon-gamma
min	Minute
MIP	Macrophage inflammatory protein
MMC	Mucosal mast cells
M-MLV	Moloney-murine leukemia virus
mRNA	Messenger RNA
n.d.	Not determined
NECA	5′-(N-Ethylcarboxamido) adenosine
NFAT	nuclear factor of activated T cells
NF-KB	Nuclear factor-kappa B
NGF	Nerve growth factor
NLR	Nod-like receptor
$P2X_7$	Purinergic receptor

PAF	Platelet-activating factor
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCMC	Peritoneal cell-derived mast cells
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PG	Prostaglandin
PI3K	Phosphoinositol 3-kinase
PIP_2	Phosphatidylinositol 4,5-biphosphate
PKA	Phosphokinase A
PLA_2	Phospholipase A_2
PKC	Phosphokinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptor
PTx	Pertussis toxin
qPCR	Quantitative PCR
Raf	Raf kinase
RANTES	Regulated on activation, normal T cell expressed and secreted
Ras	Ras kinase
RBL-2H3	Rat basophilic leukemia cell line
Rh	Recombinant human
RhoGEF	Rho-specific guanine nucleotide exchange factor
Rm	Recombinant murine
RNA	Ribonucleic acid
ROS	Reactive oxygen species

RT-PCR	Reverse transcription-PCR
s	Seconds
S1P	Sphingosine-1-phosphate
$S1P_1$	Sphingosine-1-phosphate receptor 1
$S1P_2$	Sphingosine-1-phosphate receptor 1
SA	Streptavidin
SAA	Serum amyloid A
SCF	Stem cell factor
SDF-1	Stromal cell-derived factor-1
S.E.M.	Standard error of the mean
shRNA	Small-hairpin RNA
SMC	Skin mast cells
SP	Substance P
STAT	Signal transducers and activators of transcription
TGF-β	Transforming growth factor-beta
Th	T-helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
VDCC	Voltage-dependent calcium channel
VEGF	Vascular endothelial growth factor
WASP	Wiskot-Aldrich syndrome protein
W(m)	WKYMVM

CHAPTER 1. GENERAL INTRODUCTION

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1.1 OVERVIEW

Mast cells are long-lived, tissue-resident leukocytes that are found throughout the body at locations in close contact with the external environment such as the skin, airways, and the gastrointestinal tract. Activation of mast cells through their cell surface receptors leads to selective and timely release of granule-stored and *de novo*-generated proinflammatory mediators. The multitude of mediators that can be released plays a pivotal role in determining the nature of mast cell responses, ranging from protection against pathogens to hypersensitivity and atopic diseases. This response can occur as a consequence of antigen binding to IgE molecules which occupy high affinity receptors for immunoglobulin (Ig)E (FcεRI) on the mast cell surface. Mast cell responses, however, can also be regulated by other receptors expressed on their surface. One such class of these receptors are the G protein-coupled receptors (GPCRs). The responses elicited by GPCRs are quite divergent, ranging from induction of chemotaxis and adhesion to potentiation of mast cell activation. GPCRs can not only activate mast cell function alone but also can interact with other GPCRs to augment or diminish activation. This thesis will describe chemoattractant GPCRs that are stimulatory or inhibitory in function and differentially activate mast cells depending on the particular G protein utilized for signal transduction.

1.2 MAST CELLS

Mast cells were first reported by Friedrich von Recklinghausen when he described in 1863 the presence of granulated cells in unstained connective tissues from various species, including the tail of tadpoles. They were later identified and named in 1878 by Paul Ehrlich in his doctoral thesis “Contributions to the Theory and Practice of Histological Staining” presented at Leipzig University, Germany (1). He identified them as aniline-positive granular cells in connective tissue, which he named “Mastzellen” (German word: Mast, ‘fattening’). He described these cells as being localized around developed pre-formed structures in the connective tissue, such as blood vessels, nerves, secretory ducts, sites of inflammation, and tumors. He also noted that the granules contained an undetermined chemical substance that reacted metachromatically with aniline dyes. Subsequent studies following Ehrlich’s pioneering observations revealed the biological role of mast cells in hypersensitivity reactions. Today, mast cells are no longer viewed just as mediators of allergic reactions, but also as protective cells throughout the body, functioning as critical sentinel and effector cells (2).

1.2.1 Morphology

Morphologically, mast cells have a variable shape depending on their location in the body. In loose connective tissues they are round, in close proximity to blood vessels they are elongated or ovoid, and in dermal fibers they are spindle shaped (3). Their diameter ranges between 8-20 μm (4) and the non-segmented nucleus is relatively large, measuring 4-7 μm in diameter with varying amounts of chromatin (5). The cytoplasm of mature cells contains numerous secretory granules, which can occupy up to 50-55% of the cytoplasmic space, and stain metachromatically with thiazine dyes, such as toluidine blue, showing variable patterns of scrolls, swirls, circles and lattices. When viewed by electron microscopy mast cells present numerous short and long

microvilli, numerous bulges corresponding to granules lying beneath the plasma membrane and “openings” or caveolae when granular discharge has taken place. In addition to granules, the mast cell cytoplasm contains mitochondria, Golgi apparatus, rough endoplasmic reticulum and electron-dense lipid bodies with numerous free ribosomes (6) (**Figure 1.1**).

1.2.2 Development

Mast cells originate from pluripotent $CD34^+$ stem cells derived from hematopoietic bone marrow and extramedullary tissues. They are released from bone marrow as $CD34^+$, Kit^+, $Fc\gamma RII^-$, $CD14^-$, and $CD17^-$ immature progenitors into the circulating blood (7, 8) and then are subsequently recruited into various tissues, completing their maturation under the control of locally-produced cytokines and growth factors. Interleukin (IL)-3 controls the development of progenitor mast cells and is also an important growth factor for the differentiation of rodent mast cells (9). The most important factor for maturation of human mast cells is stem cell factor (SCF), which is the ligand for receptor tyrosine kinase Kit, and is secreted by fibroblasts, stromal cells and endothelial cells (10). SCF can regulate the migration of mast cell precursors *in vivo* and the adhesion and migration of immature mast cells *in vitro*, promote the proliferation of both immature and mature mast cells, promote mast cell maturation, and modulate the expression of mast cell secretory functions (11). Pleotropic cytokines, such as IL-4, IL-6 and IL-10 also play a role in the regulation and proliferation of mast cells and together with other microenvironmental factors, they determine the final mast cell phenotype (12).

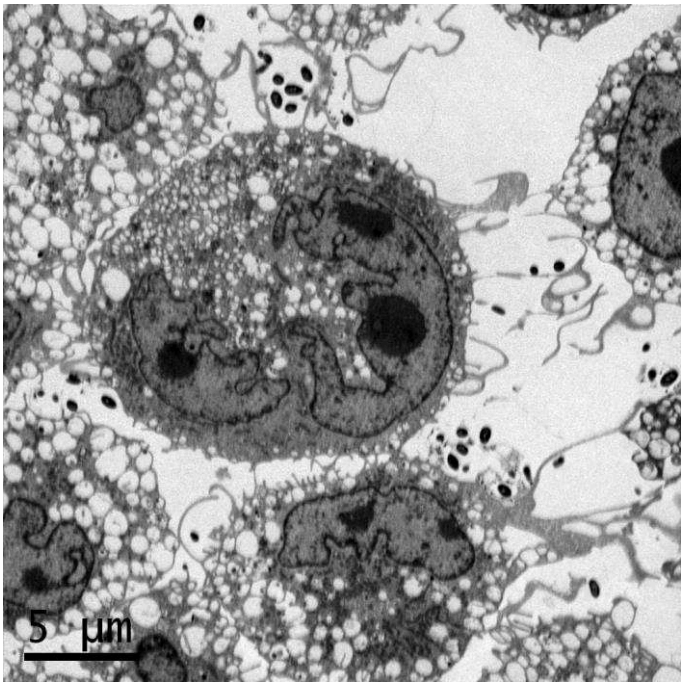

Figure 1.1 Electron micrograph of human mast cells. Laboratory of Allergic Diseases 2 (LAD2) mast cells show electron-dense secretory granules, a nucleus and numerous narrow surface folds. X 100. (Original image).

1.2.3 Heterogeneity

Mast cells exist *in vivo* and *in vitro* as a heterogeneous population of immune effector cells, subpopulations of which may be distinguished on the basis of their anatomic location, granule contents, and functional activity in response to a variety of secretagogues. In rodents, two major mast cell subpopulations have been described: mucosal-type mast cells (MMC) and connective tissue-type mast cells (CTMC) which can be differentiated using Alcian blue/safranin histochemical staining. MMC stain blue with Alcian blue, while CTMC stain red with safranin (13). MMC are mainly found in mucosa of gastrointestinal tract and in lamina propria of respiratory tract. CTMC localize mostly in the submucosa of the gastrointestinal tract, in skin, and the peritoneum. These also differ in their T cell-dependence (MMC are dependent on T cell-derived cytokines for proliferation), mediator content (proteoglycans, proteases, histamine, lipid mediators), surface antigens, and functional heterogeneity (secretagogue compound 48/80 [c48/80] induces histamine release from CTMC but not from MMC (14) (**Table 1.1**).

In humans, the distinct classification of MMC and CTMC cannot be made based on staining with dyes. Instead, immunohistochemical staining of mast cell-specific proteases tryptase and chymase is the main criterion (15, 16) of differentiation into two subpopulations: mast cells containing only tryptase (MC_T) and mast cells containing both tryptase and chymase (MC_{TC}) (17). MC_T are found in higher proportions in the lungs and mucosa of small and large intestines, while MC_{TC} are predominant in the skin and submucosa of the small and large intestines. Morphologic diversity exists between MC_T and MC_{TC}. MC_T from the intestinal mucosa and lungs have granules containing discrete scrolls (scroll-rich morphology), while MC_{TC} from the skin and intestinal submucosa have granules rimmed by incomplete scrolls (scroll-poor morphology) (18). Functionally, MC_{TC} from the skin degranulate in response to c48/80, polymyxin B, and substance P (SP), whereas MC_T from the lung and intestinal mucosa do not (19) (**Table 1.2**).

Table 1.1 Murine mucosal and connective tissue mast cells

Feature	Mucosal mast cells	Connective tissue mast cells
Tissue localization	Small intestine mucosa	Small intestine submucosa, skin, skeletal muscle, serosal surfaces
Dye binding after fixation		
Carnoy's, Mota's lead acetate, isotonic formaldehyde acetic acid	+	+
Neutral buffered formalin	-	+
Staining		
Alcian blue/safranin O	Blue	Red
Berberine sulphate	-	+
Mediators		
Histamine	<1 pg per cell	1 - 30 pg per cell
Proteoglycan	Chondroitin sulphate di-B, A, E	Heparin, Chondroitin sulphate E
Protease	MCP-1 and -2	MCP-3, -4, -5, -6, -7, and carboxypeptidase
LTC_4	>25 ng per 10^6 cells	n.d.
LTB_4	3 -12 ng per 10^6 cells	n.d.
PGD_2	1 - 5 ng per 10^6 cells	n.d.
Response to secertagouges		
IgE-antigen	+	+
A23187	+	+
Substance P	+	+
Compound 48/80	-	+
Neuropeptides	-	+

LT, leukotriene; PG, prostaglandin; MCP, mast cell protease; IgE, immunoglobulin E; n.d., not determined

Table 1.2 Human T and TC mast cells

Feature	MC_T	MC_{TC}
Tissue localization	Bronchial epithelium and subepithelium, lung alveoli, dispersed lung mast cells, small intestine mucosa, nasal mucosa epithelium, conjunctival epithelium	Skin, very few in bronchial subepithelium, small intestine submucosa, nasal mucosa subepithelium,
Mediators		
Histamine	<1 pg per cell	>10 pg per cell
Tryptase	10 pg per cell	35 pg per cell
Chymase	5 pg per cell	<0.05 pg per cell
Caroxypeptidase	-	10 – 20 pg per cell
LTC_4	10 - 80 ng per 10^6 cells	3.5 ng per 10^6 cells.
PGD_2	39 ng per 10^6 cells	43 ng per 10^6 cells
Response to secertagouges		
IgE-antigen	+	+
A23187	+	+
Substance P	-	+
Compound 48/80	-	+
Neuropeptides	-	+

LT, leukotriene; MC_T, $Tryptase^+$ mast cells; MC_{TC}, $Trypytase^+Chymase^+$ mast cells; PG, prostaglandin; IgE, immunoglobulin E

1.2.4 Distribution

The distribution and density of mast cells in the body are related largely to the content of connective tissue. They are more abundant in the dermal and subcutaneous tissue of skin, but comparatively less prevalent in liver, spleen and adrenal glands. While mast cells reside in all vascularized tissues (20), the highest numbers are observed at the interfaces of the host and environment, i.e. in skin and mucosal surfaces in the respiratory and gastrointestinal tracts (21).

1.2.5 Activation

The strategic location of mast cells within the body allows them to participate in the regulation of both innate and adaptive immune responses, as well as drive pathological hypersensitivity reactions. This versatility is reflected in numerous pathways of activation and modulation of these responses.

1.2.5.1 FcεRI-dependent activation

Stimulation via the FcεRI receptor is the best studied mechanism of mast cell activation. FcεRI is a tetrameric receptor consisting of one α chain, one β chain and two disulphide-linked γ chains. The α chain binds to IgE, whereas the β and γ chains initiate signal transduction via their immunoreceptor tyrosine-based activation motifs (ITAM). When FcεRI is aggregated upon cross-linking of bound IgE by antigen, Lyn phosphorylates the ITAM in the cytoplasmic domains of the β and γ chains and recruits Syk tyrosine kinase to the γ chain. Syk phosphorylates other tyrosine kinases, thus activating the signaling cascade. Some of these proteins include phospholipase C (PLC) which catalyzes the conversion of membrane bound phosphatidylinositol-4,5-bisphosphate (PIP_2) into inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 interacts with its receptors on the endoplasmic reticulum and triggers intracellular calcium (Ca^{2+}) release. DAG activates protein kinase C (PKC) leading to cytoskeletal

changes and degranulation. PLCγ-dependent increase of intracellular Ca^{2+} levels and activation of PKC together orchestrate the degranulation of mast cells. Phosphorylation of the linker molecule LAT initiates the Ras-Raf-Mitogen activated protein kinase/ERK kinase (MEK)-extracellular signal regulated kinase (ERK) cascade which leads to the generation of lipid mediator. The activation of PKC phosphorylates transcription factors and leads to the production of cytokines. (**Figure 1.2**)

1.2.5.2 FcεRI-independent activation

Mast cells also constructively express FcγRII (a low affinity receptor for IgG), while the expression of FcγRI (the high affinity receptor for IgG) and FcγRIII (a low affinity receptor for IgG) are induced following exposure to interferon (IFN)γ. FcγRs can contribute to mast cell activation in two ways: in an antigen-dependent manner through the binding of pathogen-specific antibodies; or independently of pathogen-specific antibodies through the action of pathogen-derived Ig-binding proteins (also known as B cell superantigens). Such activation leads to degranulation, production of lipid mediators, cytokines and chemokines (22). The tyrosine-protein kinase receptor Kit is expressed on the surface of mast cells. When this receptor binds to SCF it forms a dimer that activates its intrinsic tyrosine kinase activity, which in turn recruits and activates various signaling molecules. Subsequent propagation of signals leads to mast cell growth, differentiation, survival, chemotaxis, and cytokine production. In addition, mast cells can be activated by polybasic compounds via the activation of G proteins. Mast cells express various GPCRs; hence their function can be modulated by GPCR ligands (23, 24). They can also be activated by directly interacting with pathogens through pattern recognition receptors (PRRs), including: Toll-like receptors (TLRs), Nod-like receptors (NLRs), and the glycosylphosphatidylinositol (GPI)-anchored protein CD48 (21) (**Figure 1.3**).

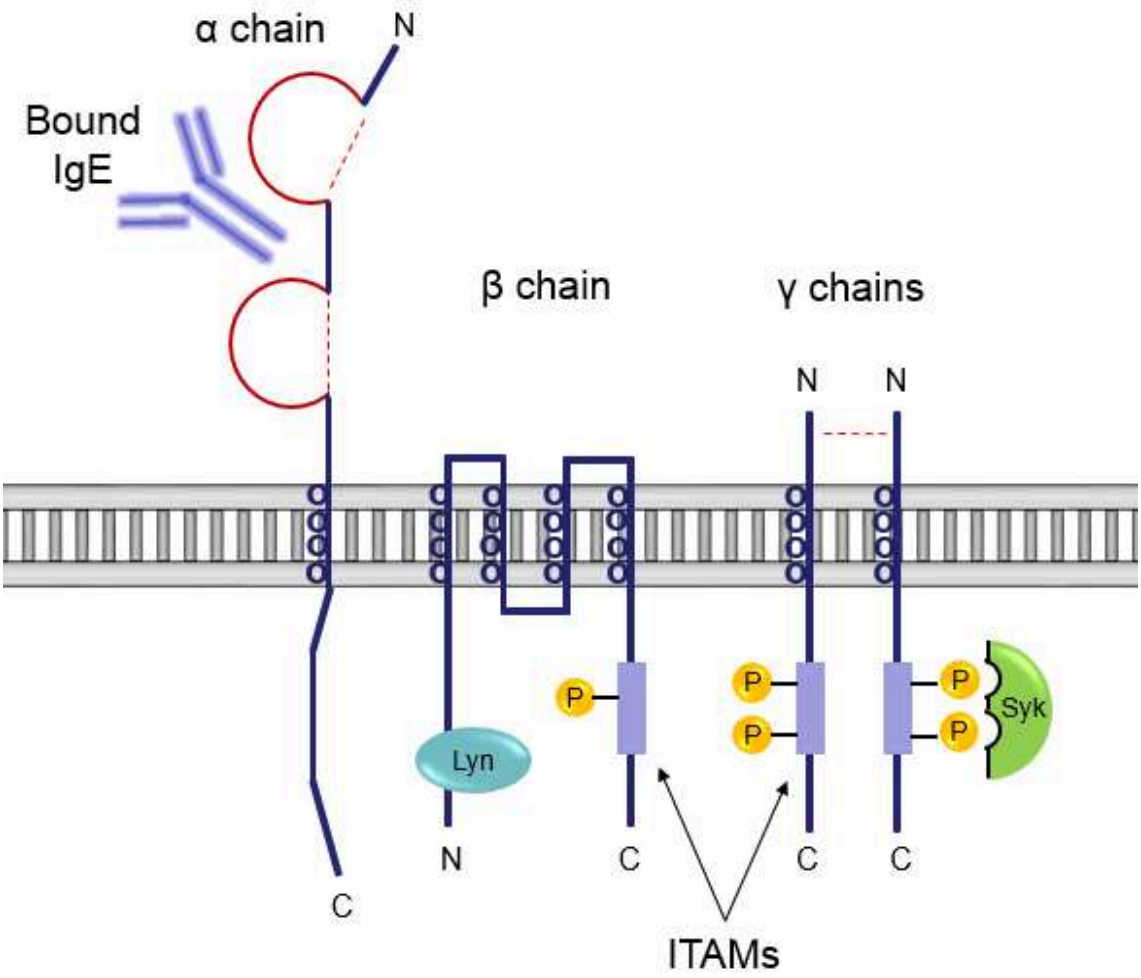

Figure 1.2 High affinity IgE receptor FcεRI. IgE binds to the Ig-like domains of the α chain. The β and γ chains mediate signal transduction via their ITAMs which recruit Lyn and Syk. (Image adapted from Kuby Immunology. Sixth Edition).

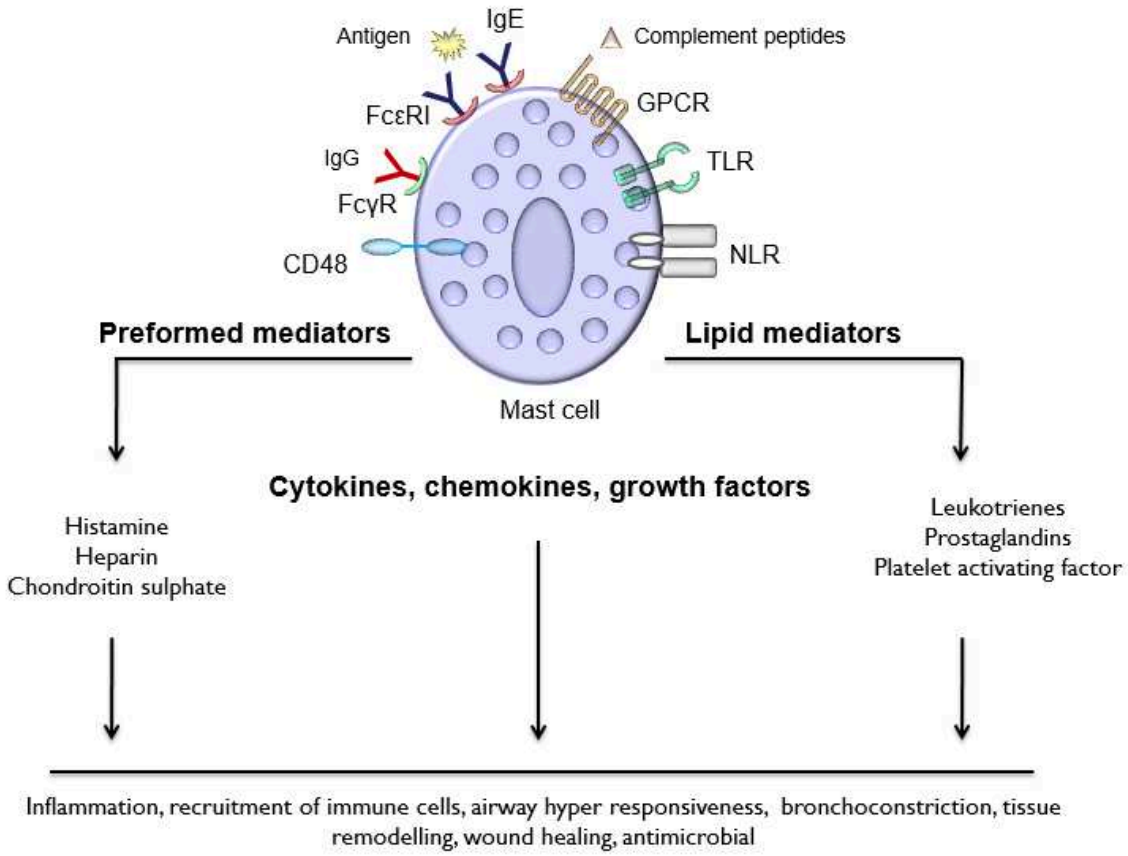

Figure 1.3 Mechanisms of mast cell activation. Mast cells express a variety of cell surface receptors that may play a role in host defense as well as hypersensitivity reactions. These include high affinity IgE receptor, FcεRI; IgG receptors, FcγRI, FcγRII, and FcγRIII; GPCRs; TLRs, NLRs, and GPI-anchored receptors that sense pathogen-associated molecular patterns. (Original image).

1.2.6 Mast cell mediators

Activation of mast cells results in the release of an impressive array of biologically active mediators, that can be divided into three major categories: (i) preformed mediators, (ii) *de novo* synthesized lipid mediators, and (iii) cytokines, chemokines and growth factors (25) (**Table 1.3**). Preformed mediators are stored in cytoplasmic granules and can be quickly released at sites of inflammation through the process of degranulation. Degranulation occurs within seconds of stimulation giving mast cell-derived mediators a temporal advantage over those produced by other immune cells. They also produce lipid-derived inflammatory mediators in the initial 5-30 minutes of activation, as gene transcription is not required for these mediators to be converted to an active form. With time, usually minutes to hours, mast cells release cytokines and chemokines that are transcribed and translated in response to stimulus. The profile of mediators produced by mast cells can significantly differ depending on the nature of stimulus and microenvironmental factors (21, 26).

1.2.6.1 Preformed mediators

Mast cell secretory granules contain biogenic amines, lysosomal enzymes, proteoglycans, proteases, and preformed cytokines. Histamine is the most well-known of these mediators and is tightly associated with proteoglycans inside the granules and released with degranulation (27). Of all the lysozomal enzymes known to be present in mast cell granules, β-hexosaminidase is associated with granules of all subpopulations and species, hence its release is frequently used as a means of quantifying the extent of degranulation in the laboratory setting (28).

Serglycin proteoglycans are also major constituents of mast cell granules (29). In rodent CTMC, heparin is the main constituent of serglycin, whereas in MMC chondroitin-

Table 1.3 Mast cell-derived proinflammatory mediators

Class of Product	Examples	Biological effects
Preformed mediators	Histamine, heparin	Increase vascular permeability, cause smooth muscle contraction
Enzymes	Tryptase, chymase, cathepsin G, carboxypeptidase	Remodel tissue matrix
Lipid mediators	Prostaglandin D_2, E_2, Leukotriene B_4, C_4	Cause smooth muscle contraction, increase vascular permeability, recruit leukocytes, stimulate mucus secretion
	Platelet-activating factor	Attracts leukocytes, amplifies production of lipid mediators, activates neutrophils, eosinophils and platelets, enhances angiogenesis and induces physiological inflammation
Cytokines	IL-3, IL-4, IL-5, IL-13	Stimulate and amplify Th_2 cell response
	IL-3, IL-5, GM-CSF	Promote eosinophil production and activation
	IL-12, IFN-γ	Stimulate and amplify Th_1 cell response
	TNF	Promotes inflammation, stimulates cytokine production by immune cells
Chemokines	CCL2, CCL3, CCL4, CXCL1, CXCL2, CXCL3, CXCL10	Attracts monocytes, macrophages and neutrophils
Others	Nitric oxide and superoxide radicals	Antimicrobial
	Antimicrobial peptides	Antimicrobial
	Osteopontin	Tissue remodeling

CCL, chemokine (C-C motif) ligand; CXCL, chemokine (C-X-C motif) ligand; GM-CSF, granulocyte macrophage-colony stimulating factor; IFN, interferon; IL, interleukin, TNF, tumor necrosis factor

sulphate is the predominant component. In contrast, serglycin in human mast cells contains both heparin and chondroitin-sulfate, in a 2:1 ratio, respectively (30). Staining of mast cells by various cationic dyes can be explained by their strong binding to serglycin present in the granules; therefore, the binding properties of these dyes can be employed to distinguish subpopulations. Granules are also a major site for stored proteases, accounting for >25% of the total mast cell protein content, with chymase, tryptase and carboxypeptidase A being the major proteases expressed (31). In addition, mast cells can also store preformed cytokines/growth factors within secretory granules, such as tumor necrosis factor (TNF), IL-4, transformation growth factor (TGF)-β (32), vascular endothelial growth factor (VEGF) (33), nerve growth factor (NGF) (34) and SCF (35).

1.2.6.2 Lipid mediators

Activated mast cells from all species have the capacity to synthesize and release lipid mediators and specifically leukotrienes and prostaglandins, which are synthesized quickly from their polyunsaturated fatty acid precursor arachidonic acid. Arachidoinic acid is mobilized from its storage sites in membrane lipids by the enzyme phospholipase A_2 (PLA_2). It is then metabolized by cyclooxygenase and lipoxygenase enzymes to generate prostaglandins, thromboxanes and leukotrienes, respectively, which affect vascular permeability, chemotaxis of immune cells, mucus production and activation of nerve cells (36). Mast cells exhibit heterogeneity in the release of lipid mediators. Rat peritoneal mast cells produce PGD_2 but little leukotriene, whereas MMC produce LTB_4 and LTC_4 along with PGD_2 (37, 38). Human lung mast cells (MC_T) synthesize nine times more leukotrienes when compared to human skin mast cells (MC_{TC}) (39).

1.2.6.3 Cytokines, chemokines and other factors

The list of cytokines and chemokines that mast cells produce following stimulation is extensive. At the present, more than thirty different cytokines have been

shown to be produced by human mast cells (40). Cytokines and chemokines act both to activate local immune cells and to promote cell recruitment to sites of infection. In addition, mast cells have also been shown to synthesize products such as antimicrobial peptides (41) and free radicals (42) that influence the immune response at epithelial surfaces.

1.2.7 Mast cell chemotaxis and adhesion

As previously mentioned, mast cells are derived from progenitors that are released from bone marrow into the circulation, and subsequently migrate into peripheral tissues, where they undergo differentiation and maturation. This recruitment depends on the presence of chemoattractants, which are produced locally at the sites of inflammation by immune cells (neutrophils, monocytes, NK cells, dendritic cells, basophils, eosinophils, B cells, and T cells) and non-immune cells (epithelial cells, fibroblasts, and keratinocytes). There are two type of mast cell migration: chemokinesis and chemotaxis. Whereas chemokinesis is a random, non-vectorial moiety, chemotaxis is a directional movement towards high concentrations of chemoattractant (43). Numerous chemoattractants have been described as inducing chemotaxis by mast cells, and will be discussed in detail in sections 1.4 and 1.5. Chemoattractants recruit mast cells by binding surface receptors that activate integrins (e.g., the receptor for fibronectin) causing adhesion to respective ligands on the vascular endothelium. Arrest of mast cell movement (similar to leukocytes) and its subsequent diapedesis through the endothelium is regulated by coordinated adhesive interactions and cytoskeletal changes in the mast cell (44). The ability of mast cells to adhere to fibronectin, collagen, and laminin plays a role in their migration into tissues (45). SCF, a main mast cell growth factor, is also a potent mediator of mast cell adhesion to fibronectin and functions through its interaction with the integrin β-1 (46).

1.2.8 Methods in mast cell research

Investigation of the biochemistry and function of mast cells has been made possible by various human and animal models available for research. These models have allowed researchers to study mast cells *in vitro*, *in vivo* as well as *ex vivo*. *In vitro* studies have used rodent and human mast cells. *In vivo* studies performed in animals such as mice and rats deficient in mast cells are discussed in following subsections.

1.2.8.1 Rodent mast cells

1.2.8.1.1 Mouse bone marrow-derived mast cells (BMMC)

BMMCs are the most widely used primary mast cell model. Bone marrow cells cultured in the presence of recombinant murine (rm)IL-3 for 3 weeks give rise to >98% pure FcεRI^{+} Kit^{+} mast cells that can be maintained in culture for 8-12 weeks. Phenotypically, these cells resemble MMC (**Figure 1.4**). The addition of rmSCF to culture skews the phenotype towards CTMC. BMMC have many advantages as they are easy to grow in large quantities and to maintain cost effectively in culture. They also prove useful in adoptive transfer technology for replenishing mast cells in mast cell-deficient animals (47).

1.2.8.1.2 Mouse peritoneal cell-derived mast cells (mPCMC)

One limitation to BMMC is their MMC phenotype; they do not respond to secretagouges and are not truly representative of a human MC_{TC} phenotype (**Table 1.1 and 1.2**). To overcome this limitation, mPCMC can be cultured in the presence of rmSCF and closely resemble CTMC. After two weeks, cultures consist of >98% pure FcεRI^{+} Kit^{low} mast cells that can be kept growing for about one month. Following that the cells stop growing but can be kept alive in rmSCF-containing medium for another 2-3 months (48). Although mPCMC respond to IgE-antigen, the relevance of this cell type is questionable since humans normally have few mast cells in the peritoneal cavity.

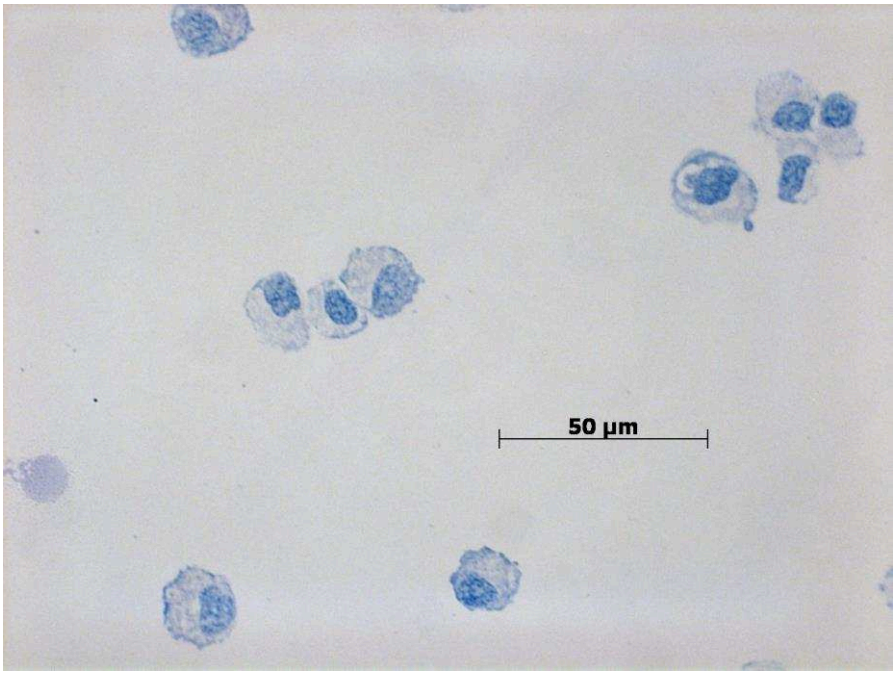

Figure 1.4 Murine mast cells cultured from bone marrow in IL-3 for 7 weeks stained with toluidine blue. Relatively few purple granules are seen, suggesting that BMMC present only small amounts of histamine and heparin. X 100. (Original image).

1.2.8.1.3 Rat basophilic leukemia-2H3 cells (RBL-2H3)

RBL-2H3 cells have been extensively used for studying IgE-FcεRI interactions, signaling pathways for degranulation and testing novel mast cell stabilizers. As these cells are adherent, they have also been used in microscopy experiments and drug screening. However, discrepancies have been observed between the results of various experiments. RBL-2H3 cells were isolated and cloned in 1978 from a histamine releasing subline of RBL-1, a basophilic leukemia cell line from Wistar rats basophilic cells that were maintained as tumors. These cells present typical characteristics of both MMC and basophils, and are an imprecise model for mast cells (49).

1.2.8.1.4 MC/9 cells

MC/9 is a cloned murine cell line with characteristics of mast cells that depends on rmIL-3 for its growth (50). It phenotypically resembles MMC isolated from a variety of sources, notably those of the lung and intestinal mucosa. MC/9 granules contain chondroitin sulphate rather than heparin. Biochemically, MC/9 cells can be stimulated by calcium ionophore A23187, concanavalin A, and IgE-hapten, as well as other agents. They can be easily grown in large numbers in suspension cultures, providing the opportunity to perform accurate biochemical analysis (51).

1.2.8.2 Mast cell-deficient mice

As the development mast cells is crucially dependent on the binding of SCF to Kit, mouse strains with alterations in the *c-Kit* gene can be mast cell-deficient and have been utilized for studying mast cell functions (52). The *c-Kit* mutant WBB6F$_1$-*Kit*$^{W/W\text{-}v}$ and C57BL/6J-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice are the most widely used models. *Kit*$^{W/W\text{-}v}$ mice have truncated and point-mutated *W* and *W*^{V} alleles, respectively, leading to reduced Kit expression, signal transduction and severe mast cell deficiency. However, as Kit is important for the development of other cell lineages, *Kit*$^{W/W\text{-}v}$ mice are also deficient in erythrocytes,

neutrophils, and certain T cells. As a result, these mice develop macrocytic anemia and neutropenia, and sterility (53). *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice have an insertion upstream of the *c-Kit* that leads to selective reduction in Kit expression and hence mast cell deficiency. These mice bear abnormalities, including splenic myeloid and megakaryocytic hyperplasia, but are generally milder than *Kit*$^{W/W\text{-}v}$ as these mice are neither anemic nor sterile, and have normal blood neutrophils (54). Both strains can be reconstituted with immature progenitor mast cells derived *in vitro* from bone marrow cells in "knock-in" models to define mast cell-specific contributions in experimental models. Engraftment of mast cells by intradermal, intraperitoneal or intravenous injections can result in mast cell numbers, which, dependent on the route of transfer and the anatomical site, can approach normal mast cell density, but often remain considerably lower with significant variability (55).

Other approaches to investigate *in vivo* functions of mast cells include the use of transgenic mice expressing Cre-recombinase under the control of "mast cell-specific" promoters. Such mast-cell-Cre mice can be crossed with other strains in which the gene of interest is "floxed" (56). When a gene sequence is "flanked by *loxP* sites" (floxed), Cre-recombinase can excise the specific segment from the genetic sequence. For example, *Mcpt5*-Cre transgenic mice (Cre expressed under the control of the *mast cell protease 5* promoter) delete *loxP*-flanked genomic DNA in mast cells alone, whereas no deletion occurs in other cell types. This approach has generated "improved" mast cell-deficient mouse model, permitting direct analyses of the extent to which mast cells represent important source of mediators that can also be derived from other cells (56).

1.2.8.3 Human mast cells

1.2.8.3.1 Primary human mast cells

Human mast cells can be cultured from $CD34^+$ progenitors isolated from peripheral or umbilical cord blood in the presence of rhSCF. Human peripheral blood

$CD34^+$ cell-derived mast cells (HuMC) are relatively mature with a condensed non-segmented nucleus and numerous granules, express FcεRI and are $tryptase^+$ $chymase^+$ (8) (**Figure 1.5**). By contrast, cord blood-derived mast cells (CBMC) are relatively immature with a segmented nucleus and less abundant granules. They are $tryptase^+$ $chymase^-$ and do not express significant amounts of FcεRI, but incubation for 5 days with IgE and rhIL-4 induces FcεRI expression (57). Human mast cells can also be isolated from tissues such as skin, lung, intestine, spleen, kidney, and uterus (58). Phenotypic differences exist between tissue-derived mast cells depending on their origin. For instance, skin mast cells are MC_{TC}, whereas lung mast cells are largely MC_T (17)

Although primary human mast cells are preferred over cell lines to study mast cell biology, current culture protocols are unable to achieve complete mast cell maturation as determined by functional studies and expression of cell surface markers. Other limitations include the time and cost associated with culturing progenitors for 6-12 weeks in the presence of various cytokines and growth factors, only to attain limited numbers of mast cells per culture. Isolation and purification of mast cells from tissues is labor intensive, requires large quantities of fresh tissue specimens and yields only 1-4 x 10^5 mast cells per gram of tissue (59). Moreover, the response of mast cells varies with time as cells used immediately after isolation usually respond better to physiological stimuli, such as IgE-antigen (P. Pundir, personal observation).

1.2.8.3.2 Human mast cell lines

Due to the scarcity of tissue mast cells and the costly purification procedures, human mast cell research mainly relies on the use of mast cell lines. Currently three human mast cell lines have been established: (i) HMC-1, (ii) LAD and (iii) LUVA.

HMC-1 is a SCF-independent, fast -growing, immature mast cell line established in 1988 from the peripheral blood of a patient with mast cell leukemia (60). In fact, two subclones, HMC1.1 and HMC1.2 have been characterized. HMC1.1 cells harbor the V560G *c-Kit* mutation, while HMC1.2 cells harbor both the V560G and D186V *c-Kit* mutations, with both making the Kit receptor active even in the absence of SCF. While these cells contain classical mast cell-associated markers such as histamine, tryptase, heparin and Kit, they lack native expression of FcεRI, limiting their functional application (61). The lack of FcεRI has led researchers to employ "less physiologic" stimuli, such as calcium ionophores and phorbol esters to assess mast cell function using HMC-1 cells.

LAD (Laboratory of allergic diseases) is a SCF-dependent, slow-growing mast cell line developed from a patient with mastocytosis and characterized in 2003. LAD1 and LAD2 cells possess many well-established mast cell markers including FcεRI and retain characteristics of mature tissue mast cells (heavily granulated, intracytoplasmic histamine and tryptase$^+$ chymase$^+$) (62) (**Figure 1.6**). Since LAD2 cells persist in long-term cultures, this offers an invaluable model for studies with mast cells. However, their widely variable doubling-time (about 1-2 weeks) and requirement for SCF-supplemented medium makes them poorly suitable for experiments requiring large number of cells.

LUVA is a new cell line characterized in 2011 which was derived from peripheral blood CD34$^+$ cells of a patient with aspirin-exacerbated respiratory disease. These cells are granulated and tryptase$^+$ chymase$^+$ and although having a normal *c-Kit*, their growth is SCF-independent. While they were initially FcεRI$^+$ and able to release mast cell mediators (63), it seems that the cell line loses its expression of FcεRI upon long term culture (Dr. T.C. Moon, personal communication, February 2013).

1.2.8.4 *In vitro* stimulation of mast cells

FcεRI-mediated activation of mast cells can be mimicked experimentally using

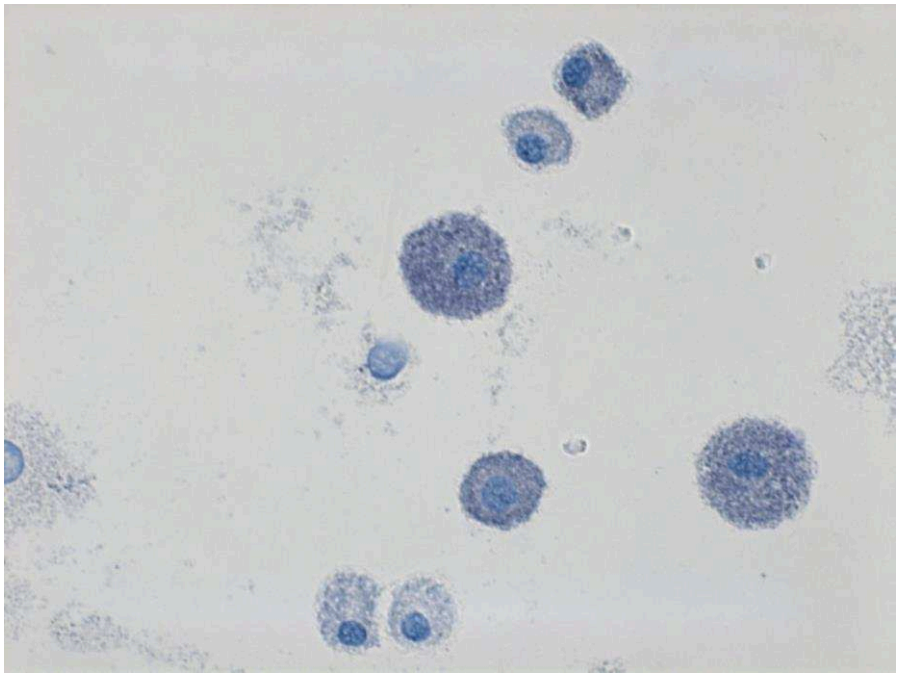

Figure 1.5 Primary human mast cells stained with toluidine blue. Peripheral blood $CD34^{+}$ cells were cultured in SCF for 7 weeks. Substantial amounts of purple granules are seen, suggesting HuMC present significant amounts of histamine and heparin. X 100. (Original image).

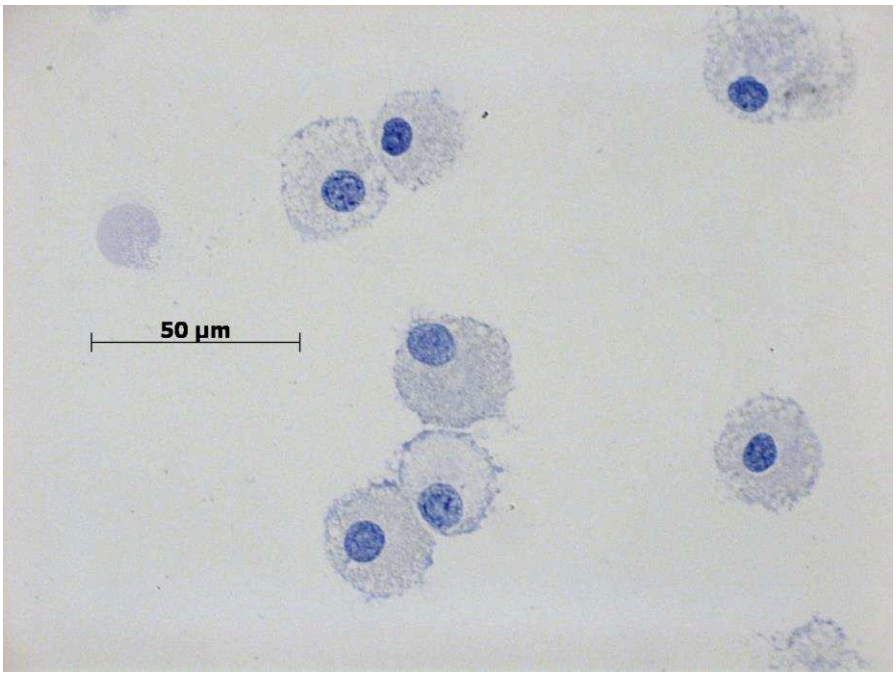

Figure 1.6 LAD2 human mast cells stained with toluidine blue. Presence of purple granules suggests LAD2 cells express histamine and heparin. X 100. (Original image).

monoclonal IgE/anti-IgE or antiserum specific for IgE which can crosslink membrane bound IgE. Calcium ionophores A23187 and ionomycin are also used as stimulants and can induce mast cell degranulation and mediator release. Other non-immunological stimuli for mast cell activation include c48/80, SP, vasoactive intestinal peptide, C5a, C3a, morphine, codeine, adenosine, and platelet-activating factor (PAF). Pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), flagellin, lipotechoic acid, peptidoglycans and double-stranded RNA are also used to stimulate mast cells to mimic their response to pathogens *in vitro.* The various stimuli have differential effects on mast cell responses depending on the type of mast cell (**Table 1.1 and 1.2**) (64, 65).

1.2.8.5 *In vivo* stimulation of mast cells

The presence/activation of human mast cells *in vivo* can be examined by various methods, including measuring the release/presence of mast cell mediators in bodily fluids, histology and immunohistochemistry of tissue biopsies, as well as determining physiological effects of ligands/drugs/stimuli with specificity for mast cells.

1.2.9 Mast cells in health

Signs and symptoms of mast cells mediators are generally associated with sneezing, wheezing, itching, and other inflammatory events often referred to as hypersensitivity. However, it has now become clear that mast cells not only release histamine, leukotrienes and prostaglandins, but also many other mediators not readily associated with inflammation. Mast cell mediators can enhance or suppress the development, survival, proliferation, migration, maturation, or function of other immune cells. For example, histamine can promote Th_1 cell activation through activation of the H_1 receptor but conversely can suppress both Th_1 and Th_2 cell activation through activation of the H_2 receptor (66). Mast cell-derived cytokines can influence the polarization and

function of T cell subpopulations (67), and IL-4, IL-5, IL-6, and IL-13 can influence B cell development and function, including IgE production (68, 69).

Mast cells are involved in immunity against extracellular parasitic infection. For example, $Kit^{W/W\text{-}v}$ mast cell-deficient mice infected with *Trichinella spiralis* show greater and persistent peak larval counts and slower worm expulsion than wild-type normal mice. Reconstitution of mast cells effectively restores protective antiparasite immune responses in these mice (70). In a model of cutaneous parasite infection by *Leishmania major*, mast cells contribute to the control of skin lesions by priming antigen-specific T cells and enhancing the recruitment of proinflammatory neutrophils, macrophages and dendritic cells (71). Mast cell function is also necessary for mice to survive infections by other pathogens including bacteria. Reconstitution in mast cell-deficient mice substantially reduces mortality and morbidity from ceacal ligation and puncture. This protection by adoptive transfer of mast cells can be inhibited by using anti-TNF neutralizing antibodies suggesting that mast cell-derived TNF induces neutrophil recruitment which is crucial for host defense against bacteria (72). Mast cells can also recognize and respond to viruses and viral products through the release of cytokines (73), recruitment of $CD8^+$ T cells, production of IFNα and the subsequent clearance of viral infection (74). Their numbers are also seen to increase in the lungs following respiratory viral infection (75). Mast cell proteases are also involved in maintaining homeostasis by degrading toxins, such as endothelin-1 (76) and snake venom (77).

Mast cells are important in homeostasis of organs that undergo continuous growth and remodeling, such as hair follicles and bones. Hair follicle cycling is severely impaired in mast cell-deficient mice, resulting in hair growth disorders (78). Mast cells produce osteopontin (79); mast cell-deficient mice have thinner, lighter and fragile femurs. Mast cell-derived IL-1, TGF-β, IL-6 and histamine can influence osteoclast recruitment and development (80). Mast cells play a major role in wound healing and

tissue repair. Growth factors, such as NGF, platelet-derived growth factor (PDGF), VEGF, fibroblast growth factor 2 (FGF2), histamine and tryptase increase vascular permeability, promote proliferation of epithelial cells and fibroblasts, initiate collagen deposition and remodeling of the matrix. Moreover, mast cells recruit other inflammatory cells to the site of injury, facilitating degradation and/or production of extracellular matrix proteins, and promoting angiogenesis by producing angiogenic mediators TNF, IL-8, FGF and VEGF (81). In many tissues, mast cells are found in close association with nerve endings, where they can communicate with neurons though the release of mediators. Histamine, serotonin and tryptase can influence neuronal activity, and in return neurons can release neuropeptides which activate mast cells (82). In the gut, mast cells and neurons play a role in maintaining homeostasis by regulating ion transport, secretion of acid, electrolytes and mucus by epithelial cells, and gastrointestinal motility (83).

1.2.10 Mast cells in disease

Mast cells are triggers of the allergic or Type I hypersensitivity responses. The importance of mast cells in allergic reactions is emphasized by their increased numbers in affected tissues. In patients with asthma, their numbers are increased following allergen exposure and are localized within the airway smooth muscle (ASM), epithelium, and mucous glands (84). This critical localization in proximity to key anatomical structures involved in asthma and evidence for direct interaction between mast cells and ASM cells (85) suggest they play a significant role in the pathophysiology of this disease. ASM can recruit mast cells by secreting CXCL8 and CXCL1, and can enhance survival and induce degranulation. Mast cells can induce TGF-β1 expression in ASM cells via release of β-tryptase, resulting in tissue remodeling (86). Mucosal mast cells under the influence of IL-4 and IL-9 are highly responsive to inhaled allergens causing

bronchoconstriction. The initial rapid release of mediators such as histamine, LTC_4 and PGD_2 contributes to the acute signs and symptoms associated with early-phase asthmatic reactions and may range from mild rhinitis to anaphylactic shock and death. These mediators induce vasodilation, contraction of the bronchial smooth muscle (producing airflow obstruction and wheezing) and increased mucosal secretions (exacerbating airflow obstruction in the lower airways and producing a runny nose). Mast cells also have a role in late-phase asthmatic reaction by mediating local accumulation and activation of T cells, dendritic cells, neutrophils, eosinophils and monocytes. Mast cell-produced Th_2 cytokines IL-4, IL-5 and IL-13 which regulate IgE synthesis by B cells, development of eosinophilc inflammation, and secretion of cytokines (TGF-β and FGF-2) associated with tissue remodeling in asthma.

Mast cells are also active modifiers of autoimmune diseases. Disease-associated increases in mast cell numbers, along with mast cell degranulation and secretion of mediators at sites of inflammation are commonly observed in many autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, type I diabetes, and bullous pemphigoid. Histamine, PAF, TNF, CXCL2, and IL-8 released within minutes of mast cell activation initiate the influx of neutrophils, thus contributing to neutrophil-driven tissue damage in autoimmune diseases. In addition, activation of mast cells through PRRs, FcεRI, and FcγR appears to be the common mechanism linking autoimmunity and allergy.

Mast cells also play pivotal roles in cardiovascular diseases. Pathological studies have shown increased presence of mast cells in perivascular tissue during atherosclerotic plaque progression, while plaque rupture is associated with myocardial infarction and stroke. Mast cells promote lipid accumulation, matrix degeneration, apoptosis, leukocyte influx into the plaque, and microvascular leakage, all resulting in plaque destabilization and rupture. Mast cell-deficient atherosclerotic $LDLr^{-/-}$ mice have

reduced atheroscelorsis compared to control mice, while repopulation of these mice with BMMC reverses the beneficial effect of mast cell absence. Mast cell numbers are also found to increase during heart failure, especially in patients with ischemic and idiopathic cardiomyopathy (87).

Mast cells play a dual role in cancer, as their presence in tumor tissue may affect prognosis both positively and negatively. Mast cell infiltrates surround tumor and secrete mediators that act as growth factors, facilitating tumor growth, angiogenesis, and metastasis. These include heparin, IL-8 and VEGF, which induce neovascularization, histamine, an immunesuppressant, mitogenic factors such as NGF, SCF, PDGF, and proteases, which disrupt the surrounding matrix and facilitate metastases. By contrast, IL-4, IL-5, IL-6 and TNF can induce apoptosis of tumor cells, thus limiting tumor growth. High mast cell density together with angiogenesis is a poor predictor of clinical outcome in colorectal cancer, lung cancer, and pancreatic cancer. In contrast, mast cell infiltration has been reported to be detrimental to tumor growth in breast cancer (88, 89).

To sum up, mast cell responses are essential in both innate and acquired immunity. They are also involved in the pathogenesis of inflammatory diseases. Mast cells are continuously communicating with each other, various immune cells and the surrounding environment in orchestrating diverse functions. These interactions require a molecular framework and a mechanism for transmission of information across the cell membrane. The molecular framework consists of receptors located in the plasma membrane, such as GPCRs, transducers of G protein signaling mechanisms.

1.3 G PROTEIN-COUPLED RECEPTORS

The 2012 Nobel Prize in chemistry recognized Drs. Lefkowitz and Kobilka for their “studies of G protein-coupled receptors (GPCRs)” (90). Their groundbreaking discoveries revealed the signaling mechanisms initiated by endogenous substances,

such as adrenalin, serotonin, histamine, dopamine, and many other hormones and neurotransmitters through a family of versatile GPCRs.

1.3.1 GPCR: Definition and basic characteristic

GPCRs are the largest and most diverse superfamily of proteins located in the plasma membrane of eukaryotic cells. They mediate the action of various stimuli by activating heterotrimeric G proteins. The human genome encodes for over 800 unique GPCRs (91) which can be grouped into five major families: (i) rhodoposin family, (ii) adhesion family, (iii) frizzled/taste family, (iv) glutamate family, and (v) the secretin family (92). The physiologic function of a fraction of these 800 GPCRs remains largely unknown and referred to as the orphan GPCRs; however, due to ongoing research efforts, the actual number of orphan receptors is decreasing (93).

1.3.2 GPCR: Structure

GPCRs are composed of seven hydrophobic transmembrane α-helices domains connected by three extracellular loops and three intracellular loops (94). The extracellular region, which binds the ligand, includes the amino terminus and the intracellular region, that interacts with G proteins, β-arrestins and other downstream effectors, has the carboxyl terminus (95). The amino terminus is frequently glycosylated and may range in size from 7 to 595 amino acids. The carboxyl terminus is usually bound to the membrane by a lipid modification, such as palmitoylation, and ranges in size from 12 to 359 amino acids. A disulphide bond between two highly conserved cysteines links the second and third extracellular loops of most GPCRs (96).

1.3.3 GPCR: Ligand interaction

Ligands bind within the transmembrane domain or extracellular binding sites of GPCR. In protease-activated receptors, the ligand is generated by proteolytic cleavage

of amino terminus of the receptor (97). The binding of a ligand on GPCR depends on two basic properties: affinity and efficacy. Affinity refers to the ability of a ligand to bind to the receptor and efficacy describes the effect of a ligand on the functional properties of the receptor and its associated signaling network. Based on these attributes, there are two types of ligands for GPCR: agonist and antagonists. Agonists are ligands that have positive efficacy and fully activate the receptor, while partial agonists induce submaximal activation of the G protein even at saturating concentrations. Inverse agonists have negative efficacy as they inhibit basal activity. Antagonists have neutral efficacy, but competitively block binding of other ligands, thus preventing any signaling (98).

1.3.4 Receptor activation: Interaction with G proteins

Binding of an agonist induces a conformational change in the receptor protein that is then transferred as a signal to its intracellular carboxyl terminus, where the GPCR interacts with G proteins. Heterotrimeric G proteins are the molecular switches that turn on signaling cascades and are composed of three subunits, guanosine diphosphate (GDP) nucleotide-binding α-subunit (Gα) and a dimer consisting of β- and γ-subunits (Gβγ) (99). In humans, there are 21 Gα, which are divided into four classes: (i) $G\alpha_s$, (ii) $G\alpha_i/G\alpha_o$, (iii) $G\alpha_q/G\alpha_{11}$, and (iv) $G\alpha_{12}/G\alpha_{13}$ (100) (**Table 1.4**), 6 Gβ and 12 Gγ subunits. In the inactive form, Gα is bound to GDP and tightly associated with Gβγ. Activation of GPCR promotes the exchange of GDP for GTP (guanosine triphosphate) and the dissociation of Gα-GTP from Gβγ dimer. Activated Gα and Gβγ subunits can modulate the activity of various effectors, which transduce the signal to different kinds of second messengers (99) (**Figure 1.7**).

1.3.5 Activated GPCR: Phosphorylation and internalization

Given the diversity of the GPCR superfamily, the general process by which a cell regulates the magnitude and duration of GPCR signaling is surprisingly well conserved.

Table 1.4 Characteristics of G protein α subunit

Family	Subtype	Gene	Expression	Effectors
$G\alpha_s$	$G\alpha_s$	Gnas	Ubiquitous	AC↑, VDCC↑
	$G\alpha_{olf}$	Gnal	Olfactory epithelium, brain	AC↑
$G\alpha_{i/o}$	$G\alpha_{i1}$	Gnai1	Wide	AC↓, GIRK↑
	$G\alpha_{i2}$	Gnai2	Ubiquitous	AC↓, GIRK↑
	$G\alpha_{i3}$	Gnai3	Wide	AC↓, GIRK↑
	$G\alpha_o$	Gnao	Neuronal, neuroendocrine	VDCC↓, GIRK↑
	$G\alpha_{t\text{-}rod}$	Gnat1	Retinal rods, taste cells	cGMP-PDE↑
	$G\alpha_{t\text{-}cone}$	Gnat2	Retinal cones	cGMP-PDE↑
	$G\alpha_{gust}$	Gnat3	Taste/brush cells	PDE↑?
	$G\alpha_z$	Gnaz	Neuronal, platelets	AC↓
$G\alpha_{q/11}$	$G\alpha_q$	Gnaq	Ubiquitous	PLCβ↑
	$G\alpha_{11}$	Gna11	Ubiquitous	PLCβ↑
$G\alpha_{12/13}$	$G\alpha_{12}$	Gna12	Ubiquitous	RhoGEF, Btk, cadherin
	$G\alpha_{13}$	Gna13	Ubiquitous	RhoGEF, cadherin

AC, adenylyl cyclase; cGMP, cyclic guanosine monophosphate; GIRK, G protein-regulated inward rectifier potassium channel; PDE, phosphodiesterase; PLC, phospholipase C; RhoGEF, Rho-specific guanine nucleotide exchange factor; VDCC, voltage-dependent calcium channel

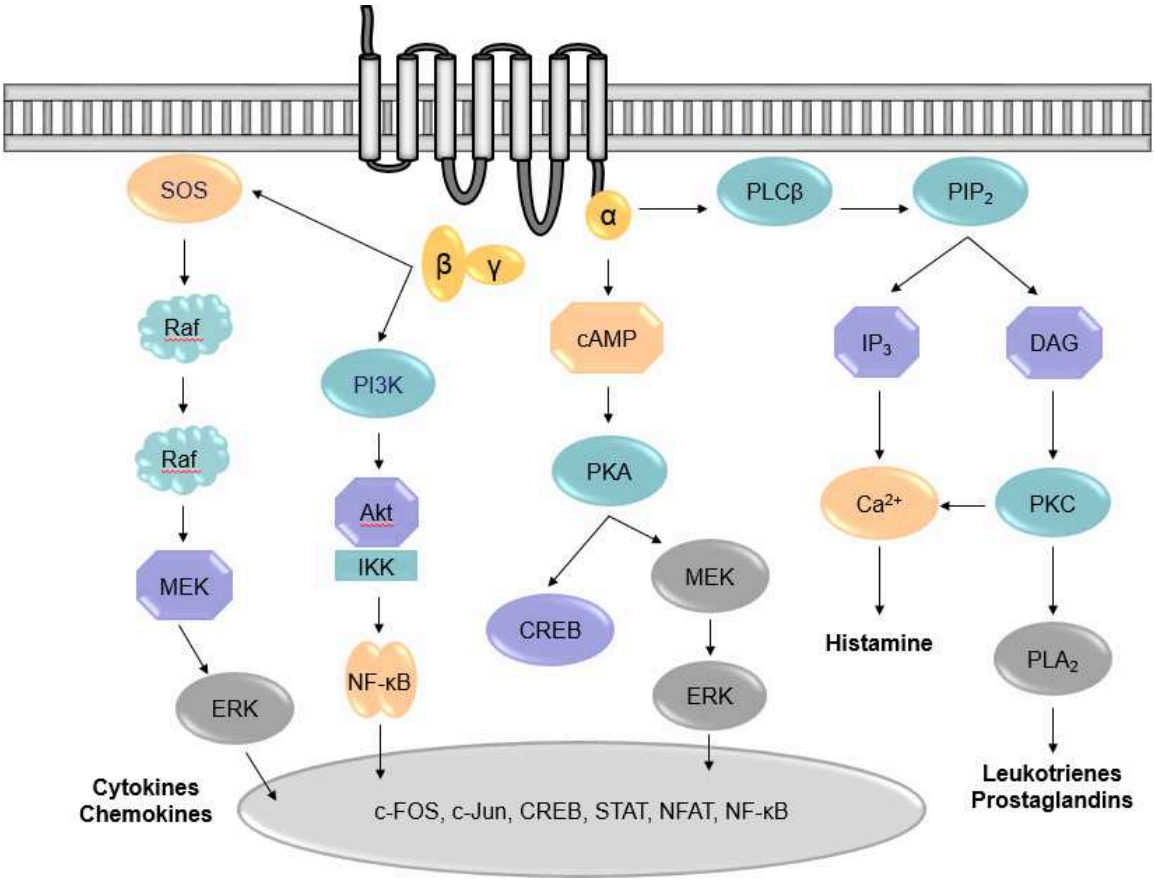

Figure 1.7 GPCR signaling cascade. The active Gα-GTP subunit stimulates phospholipase C (PLC)β, which hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). IP_3 binds to IP_3 receptors present on the surface of internal Ca^{2+} stores, mainly smooth endoplasmic reticulum, and opens Ca^{2+} channels. Release of Ca^{2+} leads to degranulation. DAG activates PKC which in turn stimulates generation of lipid mediators through cytosolic phospholipase A_2 (PLA_2). Gβγ dimer activates Ras-Raf, which phosphorylates mitogen-activated protein kinases (MAPK), and extracellular signal regulated kinases (ERK). These together with PLCβ and phosphoinositol 3-kinase (PI3K) lead to cytokine and chemokine generation. (Original image).

GPCR function can be regulated by several different mechanisms. GPCR become desensitized when exposed to an agonist for a prolonged period of time. 'Homologous' desensitization is mediated by agonist-induced activation of the same GPCR, whereas 'heterologous' desensitization is mediated by repeated stimulation of a GPCR by one agonist resulting in unresponsiveness of unrelated GPCR to other agonists. Besides, G proteins may terminate their own activation by the intrinsic GTPase activity of Gα which l hydrolyzes GTP, thereby allowing Gα-GDP to reunite with Gβγ and form an inactive heterotrimer (101). As this reaction proceeds at a slow rate (101), cofactors such as regulator of G protein signaling proteins (102) come into play that aid in desensitization.

Rapid desensitization of GPCR can also be facilitated by phosphorylation-dependent uncoupling of the receptor from heterotrimeric G proteins by the GPCR kinase (GRK)-arrestin pathway. There are seven GRKs in humans (GRK1-7) and four arrestins (arrestin 1-4). Most GPCRs are regulated by only four GRKs: GRKs 2, 3, 5, or 6, and two arrestins: arrestin-2 (β-arrestin1) and arrestin-3 (β-arrestin2) (103). Just as G proteins recognize activated GPCR, GRKs also recognize activated GPCR, which leads to receptor phosphorylation at various serine/threonine residues on the intracellular loops and the carboxyl-terminal tail (104). Upon phosphorylation by GRK, GPCR's affinity for arrestin proteins is increased, which prevents the receptor from activating additional G proteins. The β-arrestins interact with clathrin and the adaptor protein complex AP-2 and target the agonist-occupied receptors to pre-existing clathrin-coated pits for internalization. Thus GRK phosphorylation and arrestin binding result in termination of GPCR signaling, despite the continued presence of agonist (105, 106).

1.4 GPCRs AND MAST CELL FUNCTION

Host- and pathogen-derived GPCR agonists regulate innate inflammatory responses through modulating mast cell chemotaxis, survival, and activation. GPCR-

activated physiological responses are important for maintaining homeostasis, but in some cases, disregulation leads to the development of pathological conditions. Nevertheless, activation of GPCR by extracellular stimuli enhances the motile phenotype of mast cells, which results from the activation of numerous signaling cascades involving proteins, such as GTPases (107), kinases (108), and β-arrestins (109). These ultimately act in concert to regulate remodeling of the actin cytoskeleton and cell migration. In many cases, these GPCRs also control the activated state of the recruited cells (110). Chemokines are small molecular weight proteins that bind to their cognate receptors to elicit chemotaxis. The activation of receptors, such as CXCR2, plays an important role in mast cell migration, mediator release, wound healing, angiogenesis and inflammation (111). Activation of CXCR4, another chemokine receptor, on mast cells plays an important role in hematopoiesis as well as in the development, and organization of the immune system (112). Several biolipids such as sphingosine-1 phosphate (S1P), cysteinyl leukotrienes (CysLTs), and prostaglandins also regulate mast cell functions via GPCRs. $S1P_1$ receptor induces mast cell chemotaxis, whereas $S1P_2$ receptor stimulates degranulation and release of cytokines/chemokines (113). CysLT receptor 1-2 (114, 115) and prostaglandin receptors, EP 1-4, (116, 117) modulate mast cell responses in infected/inflamed tissues by increasing mast cell numbers, degranulation, and release of mediators. Neuropeptide SP releases histamine from mast cells and the effect is believed to occur through neurokinin GPCRs (118). Also, adenosine, an endogenous nucleoside released from metabolically active cells and generated exogenously by degradation of released ATP, can inhibit or enhance mediator release from mast cells depending upon which adenosine receptor is engaged on the cell surface (119).

Mast cells accumulate within hours at sites of acute inflammation. They express abundant adhesion molecules for the rapid binding to inflammation-induced counter-receptors on activated endothelial cells, and their chemoattractant receptors sense the

release of tissue-derived signals and PAMPs. Mast cells become activated and release products that in turn promote vascular permeability and leukocyte recruitment (21, 22). Numerous chemoattractants are capable of inducing chemotaxis in mast cells. Some of them, and their corresponding receptors, are summarized in **Table 1.5**. In the following sections we focus on the N-formyl peptide receptors, the complement fragment receptors, and the adenosine receptor and describe how human mast cell function is modified by these GPCRs.

1.5 THE N-FORMYL PEPTIDE RECEPTORS, THE COMPLEMENT RECEPTORS AND THE ADENOSINE RECEPTORS

The classical chemoattractants have dominated the study of leukocyte chemotaxis owing to their potency *in vitro* and *in vivo*, and to their induction by proinflammatory stimuli. In addition, most of these molecules also activate the antimicrobial functions of leukocytes, including degranulation and oxidative burst. The classical chemoattractants include N-formyl-methionyl-leucyl-phenylalanine (fMLF), C3a, C5a, and adenosine. The primary structures of chemoattractant receptors for these classical chemoatrractants have been deduced from cloned cDNAs; all of them are high affinity GPCRs (120).

1.5.1 The N-formyl peptide receptor (FPR) family

The human FPR is a GPCR that was first defined pharmacologically in 1976 as a high affinity binding site on the surface of neutrophils for the prototypical N-formylated peptide, fMLF (121). The receptor was then cloned in 1990 from cDNA library from differentiated HL-60 myeloid leukemia cells (122). Two additional genes, FPR-like 1 (FPRL1) and FPR-like 2 (FPRL2), were subsequently cloned by low-stringency hybridization using FPR cDNA as a probe (123, 124). Although all three receptors in

Table 1.5 Different mast cell chemoattractants and their receptors

Chemoattractant	Receptor	Mast cell model
SCF	c-Kit (CD 117)	BMMC, PMC, CBMC, HMC-1
IgE-antigen	FcεRI	MC/9, BMMC
S1P	SIPR1, S1PR2	BMMC, RBL-2H3, CBMC, LAD2
PGE_2	EP3	BMMC
PGD_2	DP1, DP2 (CRTH2)	BMMC
LTB_4	BLT1, BLT2	BMMC, CBMC, HMC-1
LTD_4, LTC_4	CysLT1R, CysLT2R	$CD34^+$ hematopoietic progenitors
CCL3 (MIP-1α), CCL5 (RANTES), CCL7 (MCP-3), CCL14 (HCC-1), CCL15 (MIP-1δ), CCL16 (HCC-4), CCL23 (MPIF-1)	CCR1	BMMC, LMC, CBMC, HMC-1
CCL5 (RANTES), CCL7 (MCP-3), CCL8 (MCP-2), CCL1 (eotaxin), CCL13 (MCP-4), CCL15 (MIP-1δ), CCL24 (eotaxin-2), CCL26 (eotaxin-3), CCL28 (MEC)	CCR3	BMMC, LMC, CBMC, SMC
CCL5 (RANTES)	CCR4	LMC, CBMC
CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL11 (eotaxin)	CCR5	CBMC
CCL16 (HCC-4), CCL20	CCR6	IMC
CCL19 (MIP-3β), CCL21 (6Ckine)	CCR7	BMMC, LMC, IMC
CCL1	CCR8	IMC
CXCL6 (GCP-2), CXCL8 (IL-8)	CXCR1	BMMC, LMC, CBMC, IMC
CXCL8 (IL-8)	CXCR2	CBMC, IMC
CXCL9 (MIG), CXCL10 (IP-10), CXCL11 (I-TAC)	CXCR3	BMMC, CBMC, IMC, synovial MC, HMC-1
CXCL12 (SDF-1α)	CXCR4	BMMC, CBMC, LMC, IMC

CXCL16 (GCP-2)	CXCR6	BMMC, LMC, IMC
CX3CL1 (fractalkine)	CX2CR1	BMMC, IMC
TNF	TNF receptor	PMC
TGF-β	TGFβ-I and II receptors	PMC, CBMC, HMC-1
Adenosine	Adenosine receptors	BMMC
C1q	cC1q-R, gC1q-R	HMC-1
C3a	C3aR	CBMC, SMC, HMC-1
C5a	C5aR	CBMC, SMC, HMC-1
PAF	PAF-receptor	BMMC, CBMC, HMC-1
5-Hydroxytryptamine	5-HT receptors	BMMC, HuMC
Histamine	Histamine receptor	BMMC
fMLF	n.d.	PMC
Catestatin	n.d.	HuMC, LAD2
Cathelicidin LL-37	n.d.	LAD2
β-defensins	n.d.	PMC, LAD2

BMMC, bone marrow-derived mast cells; C3a, complement component C3a; C5a, complement component 5a; CBMC; cord blood-derived mast cells; fmlp, N-formyl-methionyl-leucyl-phenylalanine; HMC-1, human mast cell line-1; HuMC, human peripheral blood $CD34^+$ cells-derived mast cells; IgE, immunoglobulin E; IL, interleukin; IMC, intestinal mast cells; LAD2, Laboratory of Allergic Diseases 2; LMC, lung mast cells; LT, leukotriene; MC/9, murine mast cell line; MCP, monocyte-specific chemokine; MIG, monokine induced by interferon gamma; MIP, macrophage inflammatory protein; PG, prostaglandin; PMC, peritoneal mast cells RANTES, regulated on activation, normal T cell expressed and secreted; RBL-2H3, rat basophilic-like human mast cell line; SCF, stem cell factor; SDF-1, stromal cell-derived factor-1; SMC, skin-derived mast cells; TGFβ, transformation growth factor beta; TNF, tumor necrosis factor; n.d., not determined.

FPR family play a critical role in the regulation of inflammatory process associated with host defense, FPRL1 will be discussed further because of its relevance to this thesis.

1.5.1.1 FPRL1 receptor

Unlike FPR which binds fMLF with high affinity (K_d < 1 nM), FPRL1 exhibits low affinity (efficacy ~1000 fold lower than FPR) for fMLF (125). FPRL1 was initially termed as ALX because of its high affinity for arachidonic acid-derived Lipoxin A_4 (LXA_4) (126). Thereafter, a number of ligands were described, making FPRL1 an exceptionally promiscuous receptor, responding to numerous ligands of different origins and structures. These include serum amyloid A (SAA), annexin 1 and its derived peptides, antimicrobial peptides, chemokines, HIV peptides, as well as synthetic peptides WKYMVM and WKYMV (W peptides), and numerous chemical compounds (C43) (127).

1.5.1.1.1 Structure

FPRL1 comprises a 350-amino acid polypeptide chain. The receptor clusters on chromosome 19q13.3, and shares 69% sequence homology with FPR (125) and 83% with FPRL2. (128). The structural domain that mediates FPRL1 binding to agonists has yet to be identified. Studies from FPR have suggested that the second and the fifth transmembrane domains may be involved in ligand binding; however, stimulation of FPRL1 by ligands which have the ability to also activate FPR, is not affected by two FPR-specific antagonists. This indicates that although certain agonists are recognized by both FPR and FPRL1, binding of these agonists to the two receptors involve different binding sites (129).

1.5.1.1.2 Expression.

FPRL1 is expressed in a large variety of cells and tissues, including monocytes, macrophages, neutrophils, T cells, B cells, microglial cells, platelets, hepatocytes,

epithelial cells, microvascular endothelial cells, fibroblasts, spleen, lung, testis, placenta, brain, astrocytoma, and neuroblastoma cells (130).

1.5.1.1.3 Signaling.

FPRL1 is coupled to $G\alpha_i$ proteins and upon activation signals through G protein-dependent signaling pathways described earlier (122, 131, 132). The synthetic peptide WKYMVM can activate FPRL1 to induce various cellular functions, such as calcium flux, chemotaxis, lipid mediator production through $cPLA_2$, MAPK, PI3K and Akt activation, and superoxide production. WRW4 peptide is an antagonist of FPRL1; it has no effect on FPR signaling but blocks activation by most FPRL1 agonists (133).

1.5.1.1.4 Biological role.

FPRL1 is strongly associated with host defense, and its ability to convey both pro- and anti-inflammatory signaling makes it an intriguing GPCR. Bacteria-derived peptides activate chemotactic and antimicrobial responses in neutrophils via FPRL1. Antimicrobial peptides produced during microbial invasion interact with FPRL1 leading to recruitment of neutrophils, monocytes, eosinophils, and T cells; therefore, amplifying innate immune response. The receptor can also act during human immunodeficiency virus (HIV)-1 infection as HIV-1 envelope proteins gp41 and gp120 are chemotactic agonists for FPRL1. Chemokine sCKβ8-1, a splice variant of CCL23, elicits FPRL1-mediated Ca^{2+} mobilization and chemotaxis in neutrophils. FPRL1/SAA have been implicated in the pathogenesis of inflammatory arthritis attributable to their ability to chemoattract monocytes, neutrophils, and T cells and stimulate production of metalloproteases, cytokines and increased expression of cytokine receptors. In contrast, FPRL1/LXA_4 has a role in anti-inflammatory pathways; it inhibits neutrophil chemotaxis, cytokine production, and superoxide generation. (128, 130, 133, 134).

1.5.1.1.5 Mast cell functions

Previous studies have shown that antimicrobial peptides are capable of chemoattracting mast cells. Antimicrobial peptides are a unique and diverse group of gene-encoded proteins, which are highly conserved in their structure, function and mechanism of action (135). The major antimicrobial peptides found in humans are defensins and cathelicidins. A variety of cathelicidins have been identified in animals (136); however, humans express only one cathelicidin, hCAP-18/LL-37 (137), produced mainly by neutrophils (138), monocytes, mast cells (139), natural killer cells, B cells (140), squamous epithelia (141), keratinocytes of inflamed skin (142), and airway epithelia (143). Apart from their direct antimicrobial functions, cathelicidins have been reported to evoke a number of mast cell activities including migration, proliferation, and cytokine and chemokine production. LL-37 stimulates mast cell degranulation and the release of PGD_2 (144-146); it also chemoattracts mast cells (146-148) and activates them to release histamine, which in turn increases vascular permeability in the skin. This requires phosphorylation of MAPK and ERK1/2 (146, 149). LL-37 induces production of the Th_1 cytokine IL-2, Th_2 cytokines IL-4 and IL-5, and proinflammatory cytokines TNF and IL-1β by human mast cells (145), suggesting that it can mediate both pro- and anti-inflammatory effects in mast cells. In addition, LL-37 has the ability to stimulate the secretion of the pruritogenic mediator IL-31 from mast cells (150), indicating a role for antimicrobial peptide-mast cell interactions in the pathogenesis of skin disorders. The majority of the studies describing mast cell activation through a GPCR have utilized pharmacological antagonists of different signaling cascades, such as pertussis toxin (inhibitor of $G\alpha_i$), wortmannin (inhibitor of PI3K), U-73122 (inhibitor of PLC), and Ro-31-8220 (inhibitor of PKC). Indeed, LL-37-mediated mast cell activation could be blocked by pertussis toxin and wortmannin (144, 146-148), indicating antimicrobial peptides potentiate mast cell responses through the G protein signal transduction. Many GPCRs

have been theorized to bind and be activated by antimicrobial peptides, but the identities of these receptors on mast cells is largely unknown (151). The ability of cathelicidins to induce Ca^{2+} mobilization in FPRL1-transfected HEK(293) cells have suggested that FPRL1 may be a receptor for LL-37 (152). However, whether LL-37 is an agonist for FPRL1 in human mast cells remains uncertain. Furthermore, the expression and function of FPRL1 in human mast cells remain elusive.

1.5.2 The complement receptors

The complement system is a biochemical cascade of the innate immune system, comprised of more than 30 humoral and membrane-associated proteins, normally present as zymogens. PRRs in the complement system, such as specific antibody, C1q, C3, mannose-binding lectin, and ficolins recognize exogenous as well as endogenous PAMPs leading to the activation of complement (153) by four different pathways: classical, alternative, lectin and extrinsic protease pathways. Each of these pathways culminate in activation of C3, the central step in complement activation. The complement proteins C3a, C4a and C5a are generated as part of the complement cascade. C3a is a 9 kiloDalton (kDa) peptide fragment released during proteolytic cleavage of the C3 α chain by a C3 convertase of the classical or the alternative pathway. C4a is a 8.7 kDa peptide released from the α chain of C4 by C2a cleavage in an early step of the classical pathway. C5a is an 11 kDa peptide released from the α chain of C5 by action of either classical or alternative pathway C5 convertase (154) (**Figure 1.8**).

C3a, C4a and C5a are potent anaphylatoxins with diverse activities on many cell types. They act as chemoattractants for neutrophils, eosinophils, monocytes, and mast cells, recruiting them to sites of injury or inflammation. They induce oxidative burst in neutrophils, histamine release from mast cells and basophils, production of eosinophil cationic protein (ECP) from eosinophils, and production of proinflammatory cytokines

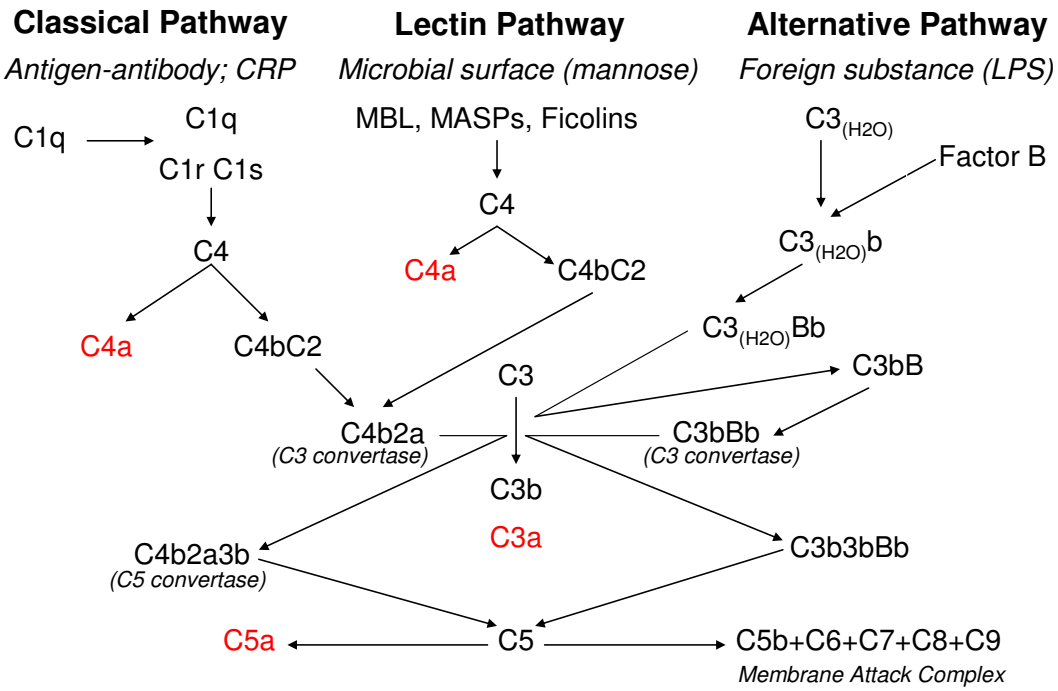

Figure 1.8 Complement activation. All three pathways that activate the complement cascade converge at the formation of a C3 convertase. This complex cleaves C3 into components C3a, C4a, and C5a and C3b, ultimately leading to pathogen opsonization, release of inflammatory mediators, and formation of terminal complement components which form the membrane attack complex. (Original image).

from monocytes, B cells and T cells. Characterization of the inflammatory activities indicate relative potency in the order of C5a > C3a > C4a on most tissues examined (155). C3a and C5a exert their pleiotropic effects by binding to a family of three receptors belonging to the GPCR superfamily. These receptors are C3a receptor (C3aR), C5a receptor (C5aR) and C5a receptor-like 2 (C5L2).

1.5.2.1 C3a receptor (C3aR)

C3aR is a membrane glycoprotein of approximately 54 kDa (156) displaying high affinity for C3a with a K_d of about 1 nM, but not for C3a desArg or C5a (157). Human platelets express a high molecular weight (95-105 kDa) variant of C3aR that binds C3a with K_d of 8×10^{-10} M.

1.5.2.1.1 Structure

C3aR contains seven transmembrane domains within its 482-amino acids sequence. It distinctively possesses a large second extracellular loop between the fourth and fifth transmembrane domain required for ligand binding. This loop contains 175 amino acid residues; in most GPCRs the corresponding extracellular loop is 30-40 amino acids long. Sulfation of tyrosine 174 in this loop is essential for binding C3a (158). The genes encoding C3aR have been mapped to q13.2-3 of chromosome 12 in humans and 6F1 in mice (159). C3aR displays 50-60% homology between various species, with 65% sequence identity between the human and murine counterparts (159).

1.5.2.1.2 Expression

C3aR is expressed on cells of myeloid origin, including monocytes/macrophages, neutrophils, eosinophils, basophils, mast cells, dendritic cells, and microglia. It is also expressed on non-myeloid cells, such as astrocytes, endothelial cells, epithelial cells, smooth muscle cells, and activated T cells.

1.5.2.1.3 Signaling

C3aR exerts its effect via coupling to the pertussis toxin-sensitive and –insensitive G proteins $G\alpha_i$ and $G\alpha_{16}$, respectively. In endothelial cells, C3aR also couples to pertussis toxin-insensitive $G\alpha_{12}$ and $G\alpha_{13}$ (160). Downstream signaling events involve activation of PI3Kγ, which in turn activates PLCβ and PLCγ, which generates IP_3 and DAG, leading to Ca^{2+} mobilization and PKC activation, respectively. PI3K can also activate the Ras-Raf-MEK-ERK1/2 cascade.

1.5.2.1.4 Biological role

C3aR mediates chemotaxis of eosinophils (161), mast cells (162), dendritic cells (163), and monocytes but not neutrophils (161). It triggers an oxidative burst in macrophages, neutrophils, and eosinophils (164). In addition, basophils (165) and mast cells (166, 167) undergo degranulation and release histamine upon C3a-C3aR interaction. It stimulates ECP production from eosinophils (168), as well as upregulation of β_2-integrins and shedding of L-selectins, thereby promoting eosinophil adhesion to endothelial and epithelial cells (169). Human monocytes and mast cells exhibit increased intracellular Ca^{2+} levels when stimulated with C3a (167). C3a also stimulates smooth muscle contraction (170), lysozyme release from immune cells (171), platelet aggregation (172) and triglyceride synthesis in adipocytes (173) .

1.5.2.2 C5a receptor (C5aR or CD88)

First cloned in 1991 (174, 175), C5aR is a membrane glycoprotein of approximately 42 kDa (176), displaying high affinity for C5a and C5a desArg. Human C5aR binds C5a with a K_d of 1 nM, and C5a desArg with 10 to 100-fold lower affinity (K_d of 412-660 nM), whereas C3a and C3a desArg are not recognized (177).

1.5.2.2.1 Structure

The C5aR gene is localized to q13.3-13.4 of human chromosome 19 (178). Murine C5aR exhibits 65% sequence homology with its human counterpart (179). The N-terminus is required for high affinity binding of C5a, but not for receptor activation (180, 181). A second distinct binding site is formed by charged residues in the second and third extracellular loops and the external faces of the transmembrane helical bundle and hydrophobic residues in the core of the C5aR. Unlike the N-terminal binding site, the second site is responsible for receptor activation (182).

1.5.2.2.2 Expression

C5aR is expressed on cells of myeloid origin, such as neutrophils, eosinophils, basophils, mast cells, dendritic cells, and monocytes, as well as on non-myeloid cells, including bronchial and alveolar epithelial cells, endothelial cells, Kupffer cells, stellate cells, astrocytes, and microglial cells (177).

1.5.2.2.3 Signaling

C5aR mainly couples to $G\alpha_i$ (183); however, ectopically expressed C5aR and C5aR on cells of the hematopoietic lineage, can also couple to pertussis toxin-insensitive $G\alpha_{16}$ (184, 185). C5a-C5aR interaction leads to activation of several components of signaling pathway, including PI3Kγ, PLCβ2 and PLD (127). It can activate the transcription factor CREB by phosphorylation at the convergence of two pathways, PI3K/Akt and ERK (186). CREB activation has been proposed to be a part of the mechanism by which C5a can delay neutrophil apoptosis and prolong an inflammatory response (187). In neutrophils, C5a causes downstream activation of p21-activated kinases (PAK), which are involved in altering cell morphology/chemotaxis, activation or potentiation of several distinct MAPK cascades and the activation of nuclear factor (NF)-κB in macrophages (188).

1.5.2.2.4 Biological role

C5aR is a powerful chemoattractant receptor for monocytes (189), neutrophils (190), eosinophils (191), basophils (192), B cells (193), and T cells (194). It also stimulates mast cell degranulation (195), chemotaxis in specific mast cell subtypes (196, 197), oxidative burst and production of reactive oxygen species (ROS) in neutrophils (198), secretion of lysosomal enzymes (199), as well as proinflammatory mediators from monocytes, eosinophils (200), and mast cells (196).

1.5.2.3 C5a receptor-like 2 (C5L2 or GPR77)

First discovered in 2000 as a putative orphan receptor, C5L2 has since been identified as a second C5a receptor (201). It is a 37 kDa protein and binds C5a with high affinity (K_d of 2.5 nM). Unlike C5aR, C5L2 binds C5a desArg with a 20-30 fold higher affinity (202).

1.5.2.3.1 Structure

C5L2 consists of 337 amino acids and shares 58% sequence homology with C5aR and 55% homology with C3aR (203). Unlike C5aR, C5L2 uses critical residues in its N-terminal domain for binding only to C5a desArg. C5L2 has been considered a binding partner for C3a, C3a desArg (204), C4a and C4a desArg; however the available data is not very convincing (205, 206). Although C5L2 has the conventional structure of a GPCR, studies have shown that C5L2 does not couple to the known G proteins. This may be due to a structural difference in the third transmembrane domain. In GPCRs, a highly conserved DRY (Aspartic acid-Arginine-Tyrosine) motif in the third transmembrane domain is important for its interaction with the corresponding G proteins. The DRY motif is DRC in C3aR and DRF in C5aR, but DLC in C5L2. Mutation of DLC in human C5L2 to DRC has been shown to increase coupling to $G\alpha_{16}$ in co-transfected HEK-293 cells and induce a functional response (206). In contrast, no functional

response occurs in RBL-2H3 cells using a C5L2-mutant where the DRY-motif and two additional regions typically involved in G protein coupling are replaced by the corresponding C5aR sequences (207).

1.5.2.3.2 Expression

C5L2 is expressed in various tissues of myeloid and non-myeloid origin and transcripts have been detected in brain, placenta, ovary, testis, spleen, and colon. Surface expression of C5L2 has been detected in lung, liver, heart, kidney, adipose tissue, skin fibroblasts, neutrophils, and immature, but not mature dendritic cells (177).

1.5.2.3.3 Signaling and biological role

C5L2 is an enigmatic receptor as available data suggests opposing roles either as a non-signaling decoy receptor for C5a and C5a desArg or as a functional receptor. In support of the first argument, no mobilization of intracellular Ca^{2+} occurs in C5L2-transfected cells after C5a administration (202, 206), in neutrophils from C5aR-deficient mice after stimulation with C5a (208), or in C5L2-expressing epithelial and myeloid cell lines (205). C5L2-transfected RBL-2H3 cells fail to degranulate with C5a or C5a desArg (202). On the contrary, C5L2 has also been demonstrated to be functional *in vitro* and *in vivo*. Neutrophils and macrophages from C5L2-deficient mice produce increased levels of TNF and IL-6 in response to C5a and LPS relative to their wildtype counterparts (209). Similarly, C5L2-deficient mice suffer from augmented inflammatory responses and higher numbers of infiltrating neutrophils in a model of pulmonary immune complex injury (210), indicating an anti-inflammatory role for C5L2. In contrast, a strong reduction in IL-1β, MIP-1α and MIP-2 levels are observed in C5L2-deficient mice compared to wildtype controls. Furthermore, C5L2-deficient mice, like C5aR-deficient mice, or mice in which either of the receptors are blocked by anti-receptor antibodies, show a higher survival

rate in mid-grade sepsis (211), supporting a proinflammatory role of C5L2. Overall, the studies point to a more complex function of C5L2 in inflammation.

1.5.2.4 The complement receptors and mast cell function

Human skin mast cells, HMC-1, HuMC and LAD2 cells express C3aR via which C3a causes sustained Ca^{2+} mobilization, degranulation and chemokine production (167, 212). C3a induces chemotaxis of HMC-1, CBMC and skin mast cells, and these responses are inhibited by receptor specific-antibodies or pertussis toxin (162, 196). The ability of C3a to induce degranulation and chemokine production involves the activation of distinct signaling pathways including PI3K, PLC, PKC and ERK1/2 (167, 212). In contrast to C3a, the effects of C5a on human mast cells are poorly understood. Human skin mast cells express C5aR and release mediators in response to C5a (212). However, lung mast cells are unresponsive to C5a, possibly due to the fact that while MC_{TC} are predominant cell types present in the skin they are the minority cell type found in the lung (17, 213, 214). It has been shown recently that MC_T cells in the lungs do not express C5aR whereas MC_{TC} cells do and that this correlates with C5a-induced degranulation in MC_{TC} cells (215). Studies with LAD2 cells have generated conflicting data; some have shown that C5a degranulates LAD2 cells (195), while others have demonstrated no degranulation with C5a (167, 216). Moreover, the expression of C5aR on the LAD2 cell surface has not been reported. C5L2 is a novel receptor for C5a (217); its expression and function in human mast cells are yet to be defined.

1.5.3. The adenosine receptors

Adenosine plays a dual role in modulating homeostasis. First, extracellular adenosine acts as an alarm molecule that reports tissue injury to surrounding tissue. Second, extracellular adenosine generates a range of tissue responses that are organ-protective thereby mediating homeostasis (218). Adenosine elicits its physiological

responses by activation of adenosine receptors. Four subtypes of adenosine receptors have been identified, termed A_1, A_{2A}, A_{2B} and A_3. They have different affinities for adenosine - A_1 and A_{2A} are high affinity receptors (Km <30 nm), whereas the A_3 and particularly the A_{2B} are low affinity receptors (Km 1-20 μM) (219, 220).

1.5.3.1 Structure

All four subtypes are members of the GPCR superfamily. Among the human adenosine receptors, the most similar are the A_1 and A_3 (49% sequence homology) and the A_{2A} and A_{2B} (59% homology).

1.5.3.2 Expression

Adenosine receptors are ubiquitously expressed throughout the body, with virtually all cells expressing one or more subtypes. They are expressed on cells of myeloid origin, such as monocytes/macrophages, dendritic cells, neutrophils, mast cells and T cells, as well as non-myeloid cells including fibroblasts, smooth muscle cells, osteoblasts, astrocytes, myocardial cells, endothelial cells, and epithelial cells (218).

1.5.3.3 Signaling

Adenosine receptors are all coupled to G proteins. A_1 and A_3 subtypes are associated with $G\alpha_i$ proteins and signal through inhibition of adenylyl cyclase and decrease in cAMP. A_{2A} and A_{2B} subtypes are associated with $G\alpha_s$ proteins and signal through stimulation of adenylyl cyclase and increases in cAMP. Adensoine receptors are also linked to various kinase pathways including PI3K, PLC, PKC and MAPKs (221).

1.5.3.4 Biological role

Adenosine receptors are crucial initiators of pro- and anti-inflammatory responses in immune cells depending on the receptor subtype involved. While activation of A_{2A} and A_{2B} limits TNF production following macrophage activation (222), A_{2A}

augments IL-10 production by macrophages exposed to E. coli (223). Adenosine promotes the recruitment of immature dendritic cells to sites of inflammation via A_1 or A_3 receptors (224, 225). At these sites adenosine induces, via A_{2A}, an anti-inflammatory dendritic cell phenotype, driving T cell response towards Th_2 profile (226). Adenosine is a potent modulator of neutrophil function; A_{2A} inhibits superoxide generation by fMLP-stimulated neutrophils (227). In addition, A_{2A} inhibits adhesion of neutrophils to the endothelium, whereas A_1 promotes neutrophil adhesion (228). A_1 and A_3 promote migration of neutrophils in response to C5a, formylated peptides and chemokines (229, 230). Adenosine receptors also regulate the inflammatory environment in asthma, ischemia, arthritis, sepsis, inflammatory bowel disease and wound healing. Lymphocyte function is potently regulated by the anti-inflammatory effects of A_{2A} in animal models of autoimmunity and ischemia (218). Due to their dual role, adenosine receptors have emerged as novel therapeutic targets in inflammation.

1.5.3.5 Mast cell function

Adenosine has been long implicated in allergic inflammation. Inhaled adenosine provokes bronchoconstriction in atopic and asthmatic individuals, but not in normal subjects (231). Furthermore, elevated levels of adenosine are found in BAL fluid and exhaled breathe condensates of allergic inflammation patients (232, 233). Although the mechanisms of how adenosine mediates bronchoconstriction remain elusive, there is overwhelming evidence that mast cell-derived cytokines, leukotrienes and histamine may have a key role in evoking airway constriction in response to adenosine (234, 235). Adenosine potentiates IgE-induced mediator release from rodent mast cells (236). Mast cell express adenosine receptor subtypes, however which specific adenosine receptor(s) mediate(s) adenosine triggered mast cell responses remains uncertain. A_1 and A_{2B} receptors in general appear to mediate proinflammatory effects by enhancing the

release of mediators. By contrast, A_{2A} and A_3 elicit anti-inflammatory effects (237). Exposure of CBMC to an A_{2A} agonist results in inhibition of tryptase release (238). In the same CBMC model, while activation of A_1 and A_3 potentiates IgE-induced degranulation, activation of A_{2B} leads to inhibition of the degranulation (239). A_{2B} stimulation of HMC-1 cells induces secretion of the Th_2 cytokines IL-4 and IL-13 (240). Studies employing human lung mast cells have demonstrated a dual effect, low concentrations of adenosine potentiate IgE-mediated degranulation, whereas higher concentrations inhibit degranulation (241, 242). Taken together, these studies illustrate that the mast cells phenotype and the adenosine receptor(s) expressed on its cell surface may alter the pathophysiological responses of adenosine. Which adenosine receptors are expressed in LAD2 human mast cells and whether adenosine can activate LAD2 cells by itself, or induce pro- or anti-inflammatory responses remain unexplored.

1.6 RATIONALE, CONCEPTUAL MODEL AND CENTRAL HYPOTHESIS

As evident, mast cells respond to chemoattractants through the corresponding cell surface GPCRs, and responses elicited by these are quite divergent, ranging from induction of chemotaxis and adhesion to mediator release. Sub-populations of GPCR can either induce mast cell activation or modify antigen-dependent mast cell activation, depending on the particular G protein utilized for relaying signal. Transduction of signals via $G\alpha_i$ alone is sufficient for the GPCRs to promote degranulation of mast cells, whether in the presence or absence of antigen, as evidenced by their sensitivity to pertussis toxin. The ability of specific GPCRs to downregulate antigen-induced mast cell activation is dependent on the fact that these receptors induce adenylyl cyclase-dependent cAMP production via $G\alpha_s$. cAMP has been demonstrated to negatively regulate mast cell function although the precise mechanisms controlling this response still remains unclear. The balance between $G\alpha_i$- and $G\alpha_s$-dependent signaling pathways keeps mast cell

degranulation in check, which is of critical importance in the pathophysiology associated with mast cell activation. It has also been proposed that the GPCRs crosstalk to produce diverse responses in mast cells. Activation of one particular signaling pathway by a GPCR can modulate signaling via another GPCR; however, such interactions are yet to be clearly defined in human mast cells (243).

To better understand the role of stimulatory and inhibitory GPCRs in human mast cell function, we utilized novel chemoattractant receptors evolutionary conserved in the immune system as our conceptual model: (i) the FPRL1 receptor which is known to couple with stimulatory $G\alpha_i$. Its expression and function in human mast cells are unknown, (ii) the C5a receptors, C5aR and C5L2. Although C5aR is known to couple with stimulatory $G\alpha_i$ in human mast cells, the expression, function and signaling mechanism of C5L2 are unknown, and (iii) the C3a receptor and the adenosine receptor(s). As the expression, function and signaling mechanism of C3a are well-defined in human mast cells, it was chosen as a model for stimulatory GPCR. The effect of adenosine receptor(s) stimulation on C3a-activated human mast cell function was investigated to determine whether the interaction between adenosine and C3a GPCRs was stimulatory or inhibitory (**Figure 1.9**).

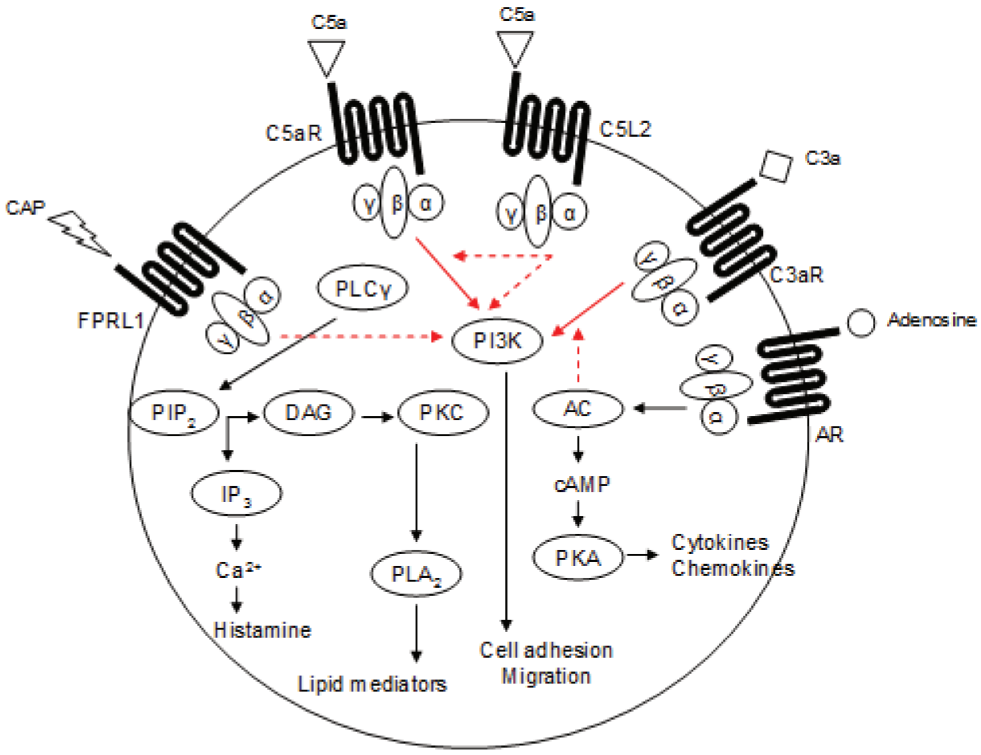

Figure 1.9 Conceptual model. Mast cells express various chemoattractant GPCRs on their cell surfaces which are either coupled to inhibitory or stimulatory G proteins. Activation of these GPCRs by respective agonists induces chemotaxis and adhesion of mast cells. In addition, specific GPCRs can differentially modulate mast cell degranulation and mediator release. The PI3K signaling pathway plays a critical role in mast cell activation through GPCRs. (Original image).

We hypothesized that human mast cells express both stimulatory and inhibitory GPCR which signal via $G\alpha_i$ or $G\alpha_s$, respectively, and that these receptors are important in chemotaxis and mast cell migration. To address this hypothesis, the following sub-hypotheses were generated:

1. Pleurocidins, novel α-cationic antimicrobial peptides, activate human mast cells through FPRL1, a $G\alpha_i$-linked stimulatory GPCR. To address this hypothesis we tested if HuMC and LAD2 cells expressed FPRL1, and if pleurocidin stimulated activation of mast cells as demonstrated by chemotaxis, adhesion, degranulation, cytokine, and chemokine production (Chapter 2).

2. C5a stimulates human mast cells through C5aR and C5L2, both $G\alpha_i$-linked stimulatory GPCRs. To address this hypothesis we tested if HuMC and LAD2 cells expressed C5aR and C5L2, and if C5a stimulated activation of mast cells as demonstrated by chemotaxis, adhesion, degranulation, cytokine, and chemokine production (Chapter 3)

3. Adenosine modulates human mast cell function through $G\alpha_s$-linked inhibitory (A_{2A}) or $G\alpha_i$-linked stimulatory (A_{2B}, A_3) GPCRs. To address this hypothesis we tested if LAD2 cells expressed adenosine receptors, and if adenosine activated human mast cells alone or in conjunction with a known stimulatory GPCR, C3aR (Chapter 4).

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CHAPTER 2. PLEUROCIDIN, A NOVEL ANTIMICROBIAL PEPTIDE, INDUCES HUMAN MAST CELL ACTIVATION THORUGH THE FPRL1 RECEPTOR

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2.1 ABSTRACT

Pleurocidins are a novel family of α-helical cationic antimicrobial peptides (CAPs) that are structurally and functionally similar to cathelicidins, one of the major CAP families. As cathelicidins stimulate mast cell chemotaxis and mediator release, we postulated that pleurocidins similarly activate mast cells. A screen of 20 pleurocidin peptides revealed that some were capable of degranulating the human mast cell line LAD2 (Laboratory of Allergic Diseases 2). Pleurocidin NRC-04 caused LAD2 to adhere, migrate, degranulate and release cysteinyl leukotrienes and prostaglandin D_2. Moreover, pleurocidin increased intracellular Ca^{2+} mobilization in mast cells and induced the production of proinflammatory chemokines such as monocyte chemotactic protein-1/C-C motif chemokine ligand 2 (CCL2) and macrophage inflammatory protein-1β/CCL4. Our evaluation of possible cellular mechanisms suggested that G proteins, phosphoinositol 3-kinase (PI3K), phospholipase C (PLC) and protein kinase C (PKC) were involved in pleurocidin-induced mast cell activation as evidenced by the inhibitory effects of pertussis toxin (G protein inhibitor), wortmannin (PI3K inhibitor), U-73122 (PLC inhibitor)

[1] P Pundir designed and performed the experiments, analyzed the data and wrote the chapter; A Catalli screened the pleurocidin peptides; C Leggiadro assisted with the electron microscopy; SE Douglas provided the pleurocidin peptides; M Kulka helped design the study and experiments, and edited the writing

and Ro-31-8220 (PKC inhibitor), respectively. We also found that human mast cells expressed the N-formyl-peptide receptor-like 1 (FPRL1) receptor and FPRL1-specific inhibitor affected pleurocidin-mediated activation of mast cells. Our finding that the novel CAP pleurocidin activated human mast cells through G protein-coupled receptor signaling suggests that this peptide might have immunomodulatory functions.

2.2 INTRODUCTION

The innate immune system is the first line of defense against invading pathogens and is dependent upon invariant receptors that recognize pathogen-associated molecular patterns. Mast cells are immune cells resident in tissues throughout the body but most common at sites exposed to the external environment, i.e. skin, mucosa of airways and intestine (1, 2, Chapter 1.2.4). Considered key players in asthma and allergy pathology, recent studies have highlighted their importance in the protection against infection (3, Chapter 1.2.9). They promote innate immune responses by rapidly and selectively producing proinflammatory mediators which are divided into three major categories: (i) preformed mediators: i.e. histamine, heparin, proteoglycans, and antimicrobial peptides; (ii) *de novo* generated lipid mediators: i.e. prostaglandins, leukotrienes, and platelet activating factor; and (iii) cytokines, chemokines, and growth factors (1, Chapter 1.2.6). Stimulation with G protein-coupled receptor (GPCR) ligands can dramatically potentiate mast cell mediator release against pathogenic organisms, thus helping to limit the pathology associated with infection (4, Chapter 1.4).

Given the need for alternate sources of multifunctional antibiotics and vaccine adjuvants, a number of novel GPCR ligands have recently been investigated for their potential to activate mast cells. Cationic antimicrobial peptides (CAPs) including defensins and cathelicidins are compounds mainly expressed in epithelial cells and cause functional changes in mast cells. Defensins and cathelicidins induce mast cell chemotaxis, degranulation, and chemokine production (5-7) in a pertussis toxin-sensitive manner which indicates that they may use GPCRs to exert their effects (6, 8). Mast cells are not only a target but are also a source of cathelicidins (9); therefore, the expression and release of CAPs, and their subsequent action is likely an important autocrine mechanism in the innate immune functions of mast cells (Chapter 1.5.1.1.5).

In 2003, 20 pleurocidin-like CAPs (NRC-01 to -20) were identified in various Atlantic flounder species and screened for antimicrobial activity (10). Structurally similar to cathelicidins, pleurocidins are mucus-derived CAPs constituting a large family of linear, positively charged, α-helical cationic peptides found in flatfish (11). Recent findings provide evidence that these proteins are important as a first line of defense in vertebrates. As with cathelicidins, pleurocidins are generated in epithelial surfaces in response to infection and exert a potent, broad-spectrum antibacterial and antifungal activity (12). Furthermore, pleurocidin has been localized to circulating cells resembling mast cells in winter flounder, where it resides in cytoplasmic granules (13). Related CAPs in the piscidin family also have been detected in fish mast cells (14). Fish mast cells are similar to their mammalian counterparts in being filled with metachromatic granules containing a diverse array of proinflammatory mediators that are released during degranulation (15). As pleurocidin can be found in mast cells of winter flounder, we were interested to see if they can initiate innate defenses in human mast cells.

Given the structural and functional similarities between pleurocidins and cathelicidin, we postulated that the pleurocidins family of CAPs also activates diverse functions of human mast cells in a G protein-dependent manner. The aims of our study were to determine: (i) the ability of pleurocidin variants in stimulating mast cell degranulation, (ii) if pleurocidins stimulate eicosanoids, cytokine and chemokine production, (iii) if pleurocidins influence mast cell adhesion and migration, and (iv) whether pleurocidin-mediated mast cell activation is GPCR-dependent. This study addressed the overall hypothesis of this thesis which is that human mast cells express both stimulatory and inhibitory GPCR which signal through $G\alpha_i$ or $G\alpha_s$, respectively, and that these receptors are important in chemotaxis and mast cell migration. We chose FPRL1 receptor as our model of activating GPCR. Although FPRL1 is known to couple with stimulatory $G\alpha_i$, its expression and function in human mast cells are unknown.

2.3 METHODS

2.3.1 Pleurocidin peptides

The amino acid sequences were predicted from nucleic acid sequences and those predicted to contain a C-terminal glycine were amidated. Peptides NRC-01 to NRC-20 were synthesized by N-9-fluorenylmethoxycarbonyl (Fmoc) chemistry at Dalton Chemical Laboratories (Toronto, ON, Canada). Peptide purity (95%) was confirmed by high-performance liquid chromatography and mass spectrometry. All peptide stocks were prepared in phosphate-buffered saline (PBS) and stored at -80°C.

2.3.2 Cell culture

LAD2 (Laboratory of Allergic Diseases 2) human mast cells (16) were cultured in serum-free medium (StemPro-34 SFM, Life Technologies, Burlington, ON, Canada) supplemented with 2 mM $_L$-glutamine, 100 U/ml penicillin, 50 µg/ml streptomycin and 0.1 µg/ml recombinant human stem cell factor (rhSCF) (Peprotech, Rocky Hill, NJ, USA). The cells were maintained at 0.1 × 10^6 cells/ml at 37°C/5% CO_2. Cells were periodically tested for expression of Kit and FcεRI by flow cytometry. Human peripheral blood $CD34^+$ cell-derived mast cells were kindly provided by Dr. Dean Befus. These cells were cultured in StemSpanSFEM (StemCell Technologies, Vancouver, BC, Canada) supplemented with 2 mM $_L$-glutamine, 100 U/ml penicillin, 50 µg/ml streptomycin, 0.1 µg/ml rhSCF, and 0.1 µg/ml rhIL-6 (Peprotech). rhIL-3 (0.03 µg/ml) was added for the first week. At 8-10 weeks, cultures consisted of >99% mast cells.

2.3.3 Degranulation

Cells were washed, resuspended in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.38 mM $Na_2HPO_4.7H_2O$, 5.6 mM glucose, 1.8 mM $CaCl_2.H_2O$, 1.3 mM

$MgSO_4.7H_2O$, 0.4% bovine serum albumin (BSA), pH 7.4) at 2.5×10^4 cells per well and stimulated for 30 min with serial dilutions (0.0001-1 µM) of each pleurocidin at 37°C/5% CO_2. β-hexosaminidase released into the supernatants and in total cell lysates solubilized with 0.1% Triton X-100 was quantified by hydrolysis of p-nitrophenyl N-acetyl-β-D-glucosamide (Sigma-Aldrich, Oakville, ON, Canada) in 0.1 M sodium citrate buffer (pH 4.5) for 90 min at 37°C/5% CO_2. The percentage of β-hexosaminidase release was calculated as a percent of total content.

In some experiments, LAD2 cells were pretreated for 30 min with wortmannin (0.1-25 µM; Sigma-Aldrich), U-73122 (0.1-25 µM; Calbiochem, Billerica, MA, USA), Ro-31-8220 (0.01-10 µM; Sigma-Aldrich) or vehicle (0.1-1% dimethyl sulfoxide); or for 2 h with pertussis toxin (0.1-10 nM, Sigma-Aldrich), WRW4 (0.5-1 µM [Biotrend Chemicals, Zurich, Switzerland]), KN-62 (10-100 nM; Sigma-Aldrich), or vehicle. Cells were then stimulated for 30 min with NRC-04 and β-hexosaminidase release was measured.

2.3.4 Electron microscopy

LAD2 cells were treated for 30 min with 1 µM NRC-04 at 37°C/5% CO_2 and fixed for 20 min in glutaraldehyde fixative (2.5% glutaraldehyde in 0.05 M cacodylate buffer with 0.1 M NaCl, pH 7.5) at room temperature. Sections were cut using a microtome and observed by using a S300N transmission electron microscope (Hitachi, Tokyo, Japan).

2.3.5 Intracellular Ca^{2+} mobilization

LAD2 cells were loaded for 30 min with 1 µM fura-2 acetoxymethyl (AM) (Life Technologies) in HEPES buffer, washed and incubated for 15 min in BSA-free HEPES at 37°C/5% CO_2. A total of 4×10^6 cells were placed in a glass-bottom culture dish under an inverted microscope (Axiovert 200, Carl Zeiss Canada Ltd., Canada). Fura-2 was excited at 340 and 380 nm alternatively. The Ca^{2+} response was recorded at 100 ms intervals using SlideBook for Stallion, version 4.26.04 software (Intelligent Imaging

Innovations, USA). In all, 1 µM NRC-04 was added at 70 s time point and 0.5 µg/ml compound 48/80 (c48/80, Sigma-Aldrich) at the256 s time point. The Ca^{2+} response of 20 randomly selected cells was analyzed for each experiment and plotted as 340/380 ratio vs. time.

2.3.6 ELISA

In each, 1×10^6 cells were stimulated for either 3 or 24 h with 0.5 µg/ml human myeloma immunoglobulin E (IgE, Calbiochem)/100 µg/ml rabibit anti-IgE (Dako, Carpinteria, CA, USA), 1 µg/ml c48/80, or 1 µM pleurocidins at 37°C/5% CO_2. Cell-free supernatants were isolated and analyzed for cysteinyl leukotrienes (CysLTs), prostaglandin D_2 (PGD_2) or chemokine using the following commercial competitive EIA kits: correlate-EIA cysteinyl leukotriene kit (Assay Designs, Ann Arbor, MI, USA), prostaglandin D_2 EIA kit (Cayman Chemicals, Ann Arbor, MI, USA), human CCL2 (C-C-motif chemokine ligand 2; monocyte chemoattractant protein-1 [MCP-1]) ELISA kit (eBioscience, San Diego, CA, USA) and quantikine human macrophage inflammatory protein (MIP)-1beta/CCL4 ELISA kit (R&D Systems, Minneapolis, MN, USA). The minimum detection limits were 78.1 pg/ml for CysLT, 200 pg/ml for PGD_2, 7 pg/ml for MCP-1 and 4 pg/ml for MIP-1β.

2.3.7 Isolation of RNA and generation of cDNA by reverse transcritption

Cells were stimulated for 3 h with 1 µM NRC-04 or 1 µg/ml c48/80. Total RNA was isolated using the Tri Reagent method (Sigma-Aldrich). In all, 1 µg of total cellular RNA was reverse-transcribed to cDNA using M-MLV Reverse Transcriptase (Life Technologies) in a 20-µl reaction mix according to the manufacturer's recommendation.

2.3.8 Reverse transcription PCR (RT-PCR)

RT-PCR was performed on a Peltier Thermal Cycler System (Bio-Rad, Hercules,

CA, USA) with Life Technologies reagents. FRPL1, $P2X_7$ and β-actin primers (**Table 2.1**) were designed using the Primer Express software (Applied Biosystems, Foster City, CA, USA). Amplification was carried out for 30 cycles of denaturation for 45 s at 94°C, annealing for 30 s at 56°C for FPRL1, $P2X_7$, and 60°C for β-actin and extension for 60 s at 72°C. PCR amplicons were analyzed by 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

2.3.9 Real-time qPCR

Gene expression was analyzed using qPCR on a StepOnePlus system (Applied Biosystems). For each qPCR assay, a total of 50 ng of cDNA was used. Primer sets (**Table 2.1**) for PCR amplifications were designed using Primer Express software. All reactions were performed in triplicate for 40 cycles as per the manufacturer's recommendation. All data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) internal controls (17) and are reported as fold-change in expression over untreated cells.

2.3.10 Cytometric bead array

In each, 1×10^6 cells were stimulated for 24 h with 1 μM NRC-04 or 1 μg/ml c48/80 at 37°C/5% CO_2. Cell-free supernatants were isolated and analyzed for human cytokine and chemokine content using the following commercial cytometric bead array kits: human inflammatory cytokine kit and human chemokine kit (BD Biosciences). The minimum detection levels for the cytokines and chemokines are IL-1β, 7.2 pg/ml; IL-6, 2.5 pg/ml; IL-10, 3.3 pg/ml; TNF, 3.7 pg/ml; IL-12p70, 1.9 pg/ml; IL-8, 0.2 pg/ml; RANTES (regulated on activation, normal T cell expressed and secreted)/CCL5, 1.0 pg/ml; monokine induced by interferon-γ (MIG)/CXCL9, 2.5 pg/ml; MCP-1/CCL2, 2.7 pg/ml; and inducible protein -10 (IP-10)/CXCL10, 2.9 pg/ml.

Table 2.1 Sequences of oligonucleotides used for PCR

Gene	Forward primer	Reverse primer	Probe: FAM/TAMRA (GAPDH: MAX/BHQ)
GAPDH	5'-TCG TGG AAG GAC TCA TGA C-3'	5'-CCA TCA CGC CAC AGT TT-3'	5'-/5MAXN/AGT CCA TGC CAT CAC TGC CAC/3IABlk_FQ/-3'
GM-SCF	5'-CAG CCC TGG GAG CAT GTG-3'	5'-ATT CAT CTC AGC AGC AGT GTC TCT A-3'	5'-/56-FAM/AGG CCC GGC GTC TCC TGA ACC/36-TAMSp/-3'
IL-1β	5'-TGA GCT CGC CAG TGA AAT GA-3'	5'-TGA GCT CGC CAG TGA AAT GA-3'	5'-/56-FAM/GAA GCT GAT GGC CCT AAA CAG ATG AAG TGC T/36-TAMSp/-3'
IL-4	5'-CTG CAA ATC GAC ACC TAT TAA TGG-3'	5'-GCA CAT GCT AGC AGG AAG AAC A-3'	5'-/56-FAM/ TCT CAC CTC CCA ACT GCT TCC CCC/36-TAMSp/-3'
IL-6	5'-AGC CAC TCA CCT CTT CAG AAC GAA-3'	5'-AGT GCC TCT TTG CTG CTT TCA CAC-3'	5'-/56-FAM/ACG GCA TCT CAG CCC TGA GAA AGG AGA/36-TAMSp/-3'
IL-8	5'-CTG GCC GTG GCT CTC TTG-3'	5'-TTG GCA AAA CTG TTT AGC ACT CC-3'	5'-/56-FAM/CAG CCT TCC TGA TTT CTG CAG CTC TGT GT/36-TAMSp/-3'
IP-10	5'-CGA TTC TGA TTT GCT GCC TTA TC-3'	5'-TGA TTA CTA ATG CTG ATG CAG GTA CA-3'	5'-/56-FAM/TGG CAT TCA AGG AGT ACC TCT CTC TAG AAC CGT/36-TAMSp/-3'
MCP-1	5'-TCT CTG CCG CCC TTC TGT-3'	5'-GCC TCT GCA CTG AGA TCT TCC-3'	5'-/56-FAM/CTG CTC ATA GCA GCC ACC TTC ATT CCC/36TAMSp/-3'
MIG	5'-GGA GTG CAA GGA ACC CCA GTA-3'	5'-TCT TTC AAG GAT TGT AGG TGG ATA GTC-3'	5'-/56-FAM/TCG CTG TTC CTG CAT CAG CAC CA/36-TAMSp/-3'
MIP-1α	5'-CTA CAC CTC CCG GCA GAT TC-3'	5'-CCG GCT TCG CTT GGT TAG-3'	5'-/56-FAM/CAG TGC TCC AAG CCC GGT GTC ATC/36TAMSp/-3'
MIP-1β	5'-CAG CGC TCT CAG CAC CAA-3'	5'-TTC CTC GCG GTG TAA GAA AAG-3'	5'-/56-FAM/CTC AGA CCC TCC CAC CGC CTG C/36-TAMSp/-3'
RANTES	5'-TCG CTG TCA TCC TCA TTG CTA -3'	5'-GCA CTT GCC ACT GGT GTA GAA A-3'	5'-/56-FAM/CTG GGA CAC CAC ACC CTG CTG C/36-TAMSp/-3'
TGF-β	5'-CTC TCC GAC CTG CAA CAG A-3'	5'-AAC CTA GAT GGG CGC GAT CT-3'	5'-/56-FAM/CCC TAT TCA AGA CCA CCC ACC TTC TGG T/36-TAMSp/-3'
TNF	5'-TCT GGC CCA GGC AGT CA-3'	5'-GCT TGA GGG TTT GCT ACA ACA TC-3'	5'-/56-FAM/ CTT CTC GAA CCC CGA GTG ACA AGC C/36T-AMSp/-3'
FPRL1	5'-TGT GAG TCT GGC CAT GAA GGT GAT-3'	5'-AGC TCG TTG GGT TAA CCA GGA TGT-3'	
P2X7	5'-TAC AGC TGC TTA GAA AGG AGG CGA-3'	5'-ACT GCC CTT CAC TCT TCG GAA-3'	
β-actin	5'-ATC TGG ACC ACA CCT TCT ACA ATG AGC TGC G-3'	5'-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC-3'	

FPRL1, N-formyl-peptide receptor 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; IP-10, inducible protein-10; MCP-1, monocyte chemotactic protein-1; MIG, monokine induced by interferon-γ; MIP, monocyte inflammatory protein; RANTES, regulated on activation, normal, T cell-expressed, and secreted; TGF-β, transforming growth factor-beta; TNF, tumor necrosis factor.

2.3.11 Adhesion assay

Maxisorp 96-well plates (NUNC, Naperville, IL, USA) were coated for 16 h with 10 µg/ml human fibronectin (Sigma-Aldrich) in PBS at 4°C, washed three times with PBS, blocked for 1 h with 3% BSA in HEPES buffer at 37°C/5% CO_2, then washed three times with HEPES buffer. LAD2 cells were washed with HEPES buffer, suspended at 1 × 10^6 cells/ml, and labeled for 20 min with 5 µM calcein-AM (Life Technologies) at 37°C/5% CO_2. After labeling, cells were washed and resuspended at 1 × 10^6 cells/ml in HEPES buffer. Cell suspension (5 × 10^4 cells) ± HEPES buffer containing 1 µM NRC-04 or 0.1 µg/ml SCF was added and incubated for 2 h at 37°C/5% CO_2. After incubation, non-adherent cells were washed away with warm HEPES buffer. Fluorescence emission at 530 nm (485 nm excitation) was measured using a fluorescence plate reader (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, Nepean, ON, Canada). The number of adhered cells was calculated from a standard curve consisting of increasing LAD2 cell numbers.

2.3.12 Chemotaxis assay

Chemotaxis was performed using a 96-well monocyte cell migration kit (Calbiochem) with slight modifications. LAD2 cells were incubated overnight in SCF-free media, washed with HEPES buffer and suspended at 0.25 × 10^6 cells/ml. Agonists (0.1 µg/ml SCF or 1 µM NRC-04) were added to the lower chamber of the 96-well tray and cell suspension (2.5 × 10^4 cells) was added to the insert (upper chamber). Cells were allowed to migrate towards the agonists for 6 h at 37°C/5% CO_2. After incubation, the insert was washed twice with warm HEPES buffer. Cells were labeled for 30 min with 5 µM calcein-AM at 37°C/5% CO_2. After labeling, the cells that had migrated across the insert membrane were lysed using 0.1% Triton X-100. Fluorescence emission was

measured at 530 nm (485 nm excitation). The number of migrated cells was calculated from a standard curve consisting of increasing LAD2 cell numbers.

2.3.13 Cell viability assay

Cells were pretreated with 1 μM NRC-04 for 30 min, 3 or 24 h, followed by incubation for 2 h with resazurin dye (In vitro toxicological assay kit, Sigma-Aldrich) at 37℃/5% CO_2. Fluorescence emission was measured at 590 nm (560 nm excitation).

2.3.14 Flow cytometric analysis

LAD2 cells were washed, suspended at 2 × 10^5 cells/ml in 0.1% BSA/PBS and incubated for 1 h with a phycoerythrin (PE)-anti-FPRL1 antibody or a PE-isotype control antibody (R&D Systems) at 4℃. Cells were washed twice, resuspended in 0.1% BSA/PBS and analyzed on a FACSArray (BD Biosciences).

2.3.15 FRET analysis

FPRL1-Gα15-NFAT-*bla* CHO-K1 cells (Life Technologies) were cultured in growth medium (DMEM (high-glucose), with GlutaMAX™ (Life Technologies) supplemented with 10% dialyzed fetal bovine serum, 0.1 mM NEAA, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml zeocin, 5 μg/ml blasticidin and 600 μg/ml geneticin). Cells were washed, resuspended in plating medium (DMEM [high-glucose], with GlutaMAX™ + 10% charcoal-stripped fetal bovine serum) and plated at 5000 cells/well in a 384-well plate; and incubated at 37℃/5% CO_2 for 16 h. After incubation the plating media was removed and replenished with assay medium (DMEM [high-glucose], with GlutaMAX™). Cells were pretreated for 2 h with the antagonists (WRW4 or pertussis toxin) followed by stimulation for 4 h with either 5 nM WKYMVM (W(M)) peptide (Phoenix Pharmaceuticals, Burlingame, CA, USA) or 1 μM NRC-04 at 37℃/5% CO_2. Following stimulation, cells were loaded in the dark for 2 h at room

temperature with LiveBLAzer^{TM}-FRET B/G substrate (Life Technologies). Fluorescence emissions were measured at 460 nm (409 nm excitation, Blue channel) and 530 nm (409 nm excitation, Green channel).

2.3.16 Statistical analysis

Each experiment was performed at least three separate times. Statistical significance was determined by two-tailed Student's paired *t*-test and $P<0.05$ was considered significant. The results are shown as mean ± S.E.M.

2.4 RESULTS

2.4.1 Effect of pleurocidin peptides on mast cell degranulation

Twenty different pleurocidin peptides (NRC-01 to NRC-20) were screened for their ability to induce human mast cell degranulation using a degranulation assay. The peptides differ in their total length and amino acid sequence (**Table 2.2**). Following stimulation of LAD2 cells with various concentrations of each of the pleurocidin peptides, we observed that not all were capable of degranulating LAD2 cells, as assessed by the release of β-hexosaminidase enzyme, a marker of histamine release (Chapter 1.2.6.1). Only 11 of the tested peptides, including NRC-02, -03, -04, -07, -10, -12, -13, -16, -17, -18, and -20, induced significant degranulation at a concentration of 1 μM (**Figure 2.1A**).

To determine whether these peptides had any cytotoxic effects on mast cells, we incubated cells with 1 μM of each peptide and measured the metabolic activity of cells using the resazurin assay. There was no change in cell metabolic activity following 30 min, 3, or 24 h stimulation (data not shown).

Table 2.2 Amino acid sequences and lengths of the pleurocidin peptides tested

Code	Amino-acid sequence	No. of residues
NRC-01	GKGRWLERIGKAGGIIIGGALDHL-NH2	24
NRC-02	WLRRIGKGVKIIGGAALDHL-NH2	20
NRC-03	GRRKRKWLRRIGKGVKIIGGAALDHL-NH2	26
NRC-04	GWGSFFKKAAHVGKHVGKAALTHYL-NH2	25
NRC-05	FLGALIKGAIHGGRFIHGMIQNHH-NH2	24
NRC-06	GWGSIFKHGRHAAKHIGHAAVNHYL-NH2	25
NRC-07	RWGKWFKKATHVGKHVGKAALTAYL-NH2	25
NRC-08	RSTEDIIKSISGGGFLNAMNA-NH2	21
NRC-09	FFRLLFHGVHHGGGYLNAA-NH2	19
NRC-10	FFRLLFHGVHHVGKIKPRA-NH2	19
NRC-11	GWKSVFRKAKKVGKTVGGLALDHYL-NH2	25
NRC-12	GWKKWFNRAKKVGKTVGGLAVDHYL-NH2	25
NRC-13	GWRTLLKKAEVKTVGKLALKHYL-NH2	23
NRC-14	AGWGSIFKHIFKAGKFIHGAIQAHND-NH2	26
NRC-15	GFWGKLFKLGLHGIGLLHLHL-NH2	21
NRC-16	GWKKWLRKGAKHLGQAAIK-NH2	19
NRC-17	GWKKWLRKGAKHLGQAAIKGLAS-NH2	23
NRC-18	GWKKWFTKGERLSQRHFA-NH2	18
NRC-19	FLGLLFHGVHHVGKWIHGLIHGHH-NH2	24
NRC-20	GFLGILFHGVHHGRKKALHMNSERRS-NH2	26

2.4.2 Mast cell degranulation in response to pleurocidin NRC-04 is concentration- and time-dependent

Out of the 11 pleurocidin peptides that activated mast cell degranulation, NRC-04 stimulated the highest amount of degranulation and it was chosen for further studies. NRC-04 degranulated LAD2 cells in a concentration-dependent manner, with maximum degranulation (58.36 ± 3.23%) at 1 μM (**Figure 2.1B**). NRC-04 also notably caused degranulation of human peripheral blood $CD34^+$ cell-derived mast cells (HuMC, **Figure 2.1B**); however, these cells had a weaker response (21.89 ± 1.1%) to NRC-04 when compared with LAD2 cells. We further characterized the kinetics of NRC-04-mediated LAD2 degranulation and found that degranulation reached a maximum (71.07 ± 3.03%) within 5 min of stimulation (**Figure 2.1C**). Since the degranulation reached its maximum by 5 min, we evaluated the effect of NRC-04 at shorter time periods. NRC-04 induced gradual degranulation in LAD2 cells from 1-5 min (**Figure 2.1D**).

To study the morphological changes associated with pleurocidin activation, we used electron microscopy to analyze intracellular changes in granularity and tomography. As shown in **Figure 2.1E**, stimulation with NRC-04 caused marked changes in the surface configuration of LAD2 cells, with loss in membrane integrity and the appearance of pores leading to the interior of the cell.

2.4.3 Pleurocidin NRC-04 induces intracellular calcium mobilization

As increase in intracellular Ca^{2+} is necessary for the induction of mast cell degranulation, we next tested whether NRC-04 had the capacity to mobilize intracellular Ca^{2+}. LAD2 cells loaded with the Ca^{2+}-sensing fluorescent dye fura-2 AM showed a rapid increase in intracellular Ca^{2+}, reaching a peak within 60 s after NRC-04 exposure. As expected, c48/80 (positive control) caused additional Ca^{2+} influx (**Figure 2.2A, B**).

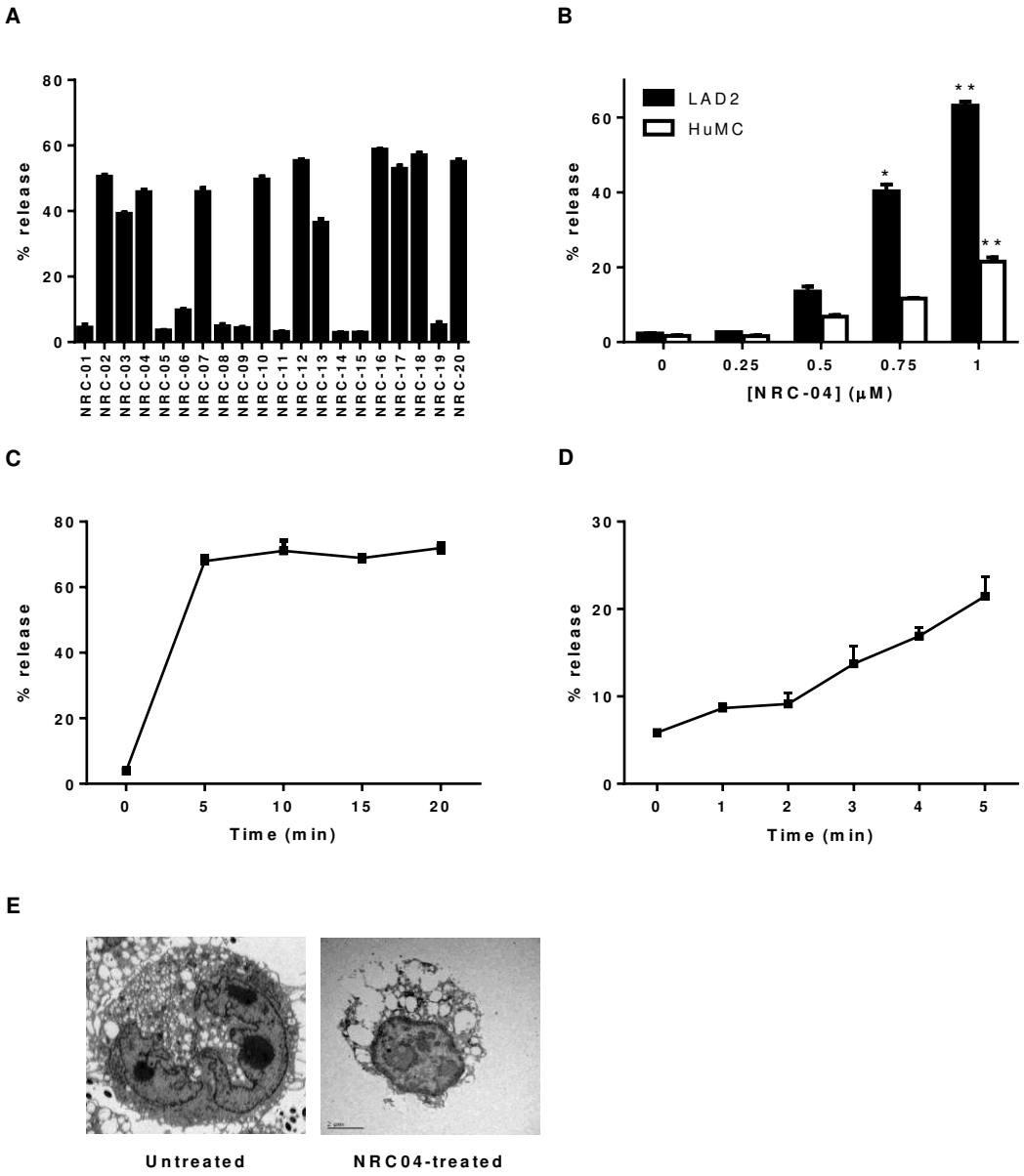

Figure 2.1 Pleurocidin peptides degranulate human mast cells. (**A**) LAD2 cells were stimulated for 30 min with 1 µM pleurocidin peptides NRC-01 to NRC-20 and % β-hexosaminidase release was measured (n=4). (**B**) Human peripheral blood $CD34^+$ cell-derived mast cells (HuMC) and LAD2 cells were stimulated for 30 min with NRC-04 at indicated concentrations or (**C-D**) LAD2 cells were stimulated with 1 µM NRC-04 for indicated times and β-hexosaminidase release was measured (n=3). (**E**) Electron micrograph showing morphological changes in LAD2 cells either unstimulated (left) or stimulated (right) for 30 min with 1 µM NRC-04. * $P<0.05$, ** $P<0.01$ compared with untreated.

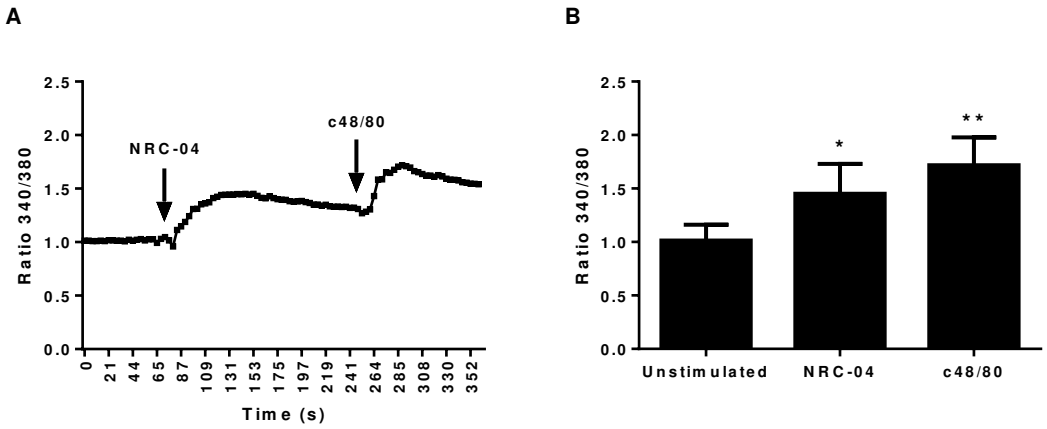

Figure 2.2 Pleurocidin NRC-04 mediates intracellular Ca^{2+} mobilization. (**A**) Fura-2 AM loaded LAD2 cells were stimulated with 1 µM NRC-04 at time point 70 s followed with 0.5 µg/ml c48/80 at time point 256 s and mobilization of intracellular Ca^{2+} was measured (n=4). (**B**) Calcium response in 20 randomly selected individual LAD2 cells reported as mean ± S.E.M. of ratio 340/380 (n=4). * $P<0.05$, ** $P<0.01$ compared with untreated.

2.4.4 Pleurocidin NRC-04 induces the release of lipid mediators CysLTs and PGD_2

Following degranulation, activated mast cells release arachidonic acid-derived lipid mediators (Chapter 1.2.6.2). As NRC-04 induced mast cell degranulation, we examined its ability to initiate the release of leukotrienes and prostaglandins. NRC-04 induced significant release of CysLTs (147.11 ± 4.55 pg/ml) and PGD_2 (935.48 ± 75.6 pg/ml) from LAD2 cells. IgE/anti-IgE stimulated similar amounts of CysLTs and PGD_2 release while the G protein agonist c48/80 had no significant effect **(Figure 2.3A, B**). Although LAD2 cells showed high spontaneous release of PGD_2, this was not attributable to any cell medium component as shown by low PGD_2 concentrations in the cell-free medium control (**Figure 2.3B)** and NRC-04 in medium alone control (data not shown).

2.4.5 Pleurocidin NRC-04 stimulates gene expression and protein production of chemokines

We further measured the expression of cytokines and chemokines by LAD2 cells using qPCR analysis. Although no effect was observed on mRNA expression of cytokines (**Figure 2.4A**), mRNA expression levels of chemokines MCP-1/CCL2 and MIP-1β/CCL4 increased significantly (**Figure 2.4B**). In addition to the cytokines and chemokines examined in **Figure 2.4A, B**, we also tested if NRC-04 induced the expression of IL-4, IL-17, granulocyte macrophage colony-stimulating factor (GM-CSF), IP-10/CXCL10, IL-1β, MIG/CXCL9, RANTES/CCL5, or IL-8. There was no change in the mRNA expression levels of these mediators following NRC-04 stimulation (data not shown). After observing the enhanced expression of MCP-1 and MIP-1β, the stimulatory effects on protein production of the respective chemokines was confirmed using ELISA,

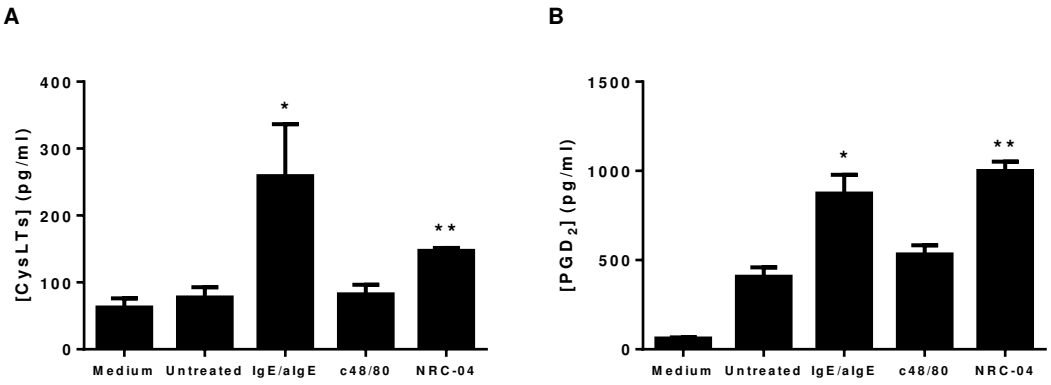

Figure 2.3 Pleurocidin NRC-04 mediates the release of lipid mediators. LAD2 cells were stimulated with 0.5 µg/ml IgE/100 µg/ml anti-IgE, 1 µg/ml c48/80, or 1 µM NRC-04. After 3 h treatment, the levels of (**A**) CysLTs, (**B**) PGD_2 in cell-free supernatants was measured by enzyme immunoassay (n=5). * $P<0.05$, ** $P<0.01$ compared with untreated.

showing that NRC-04 enhanced the production of both MCP-1/CCL2 (**Figure 2.4C**) and MIP-1β/CCL4 (**Figure 2.4D**). Interestingly, the effect of NRC-04 on chemokine production was comparable to that of IgE/anti-IgE, but considerably lower than c48/80.

To further support our hypothesis that NRC-04 preferentially activates chemokine production, we performed CBA for IL-12p70, TNF, IL-10, IL-6, IL-1β, IP-10/CXCL10, MCP-1/CCL2, MIG/CXCL9, RANTES/CCL5, and IL-8. NRC-04 induced the production of MCP-1/CCL2 only (data not shown). We next evaluated the effect of NRC-04 on HuMC and observed no production of TNF, MCP-1/CCL2, or MIP-1β/CCL4 (**Figure 2.4E**). In comparison, NRC-05 did not result in mast cell degranulation, and also failed to stimulate the production of TNF, MCP-1/CCL2 and MIP-1β/CCL4 by LAD2 cells. These cells were responsive to c48/80 which was used a positive control (**Figure 2.4F**).

2.4.6 Pleurocidin NRC-04 induces mast cell adhesion and chemotaxis

Activation of mast cell degranulation in response to infection requires mast cell migration and accumulation at sites of infection (Chapter 1.2.7). As CAPs evoke mast cell adhesion, chemotaxis, and subsequent degranulation (Chapter 1.5.1.1.5), we evaluated whether NRC-04 could also promote these functions. Comparable with SCF, NRC-04 activated LAD2 cells to adhere to fibronectin, an extracellular matrix protein (**Figure 2.5A**). Since adhesion is closely related to cytoskeletal reorganization and migration, we measured LAD2 chemotaxis to either SCF or NRC-04. Results showed that similar to SCF, NRC-04 activated LAD2 chemotaxis (**Figure 2.5B**).

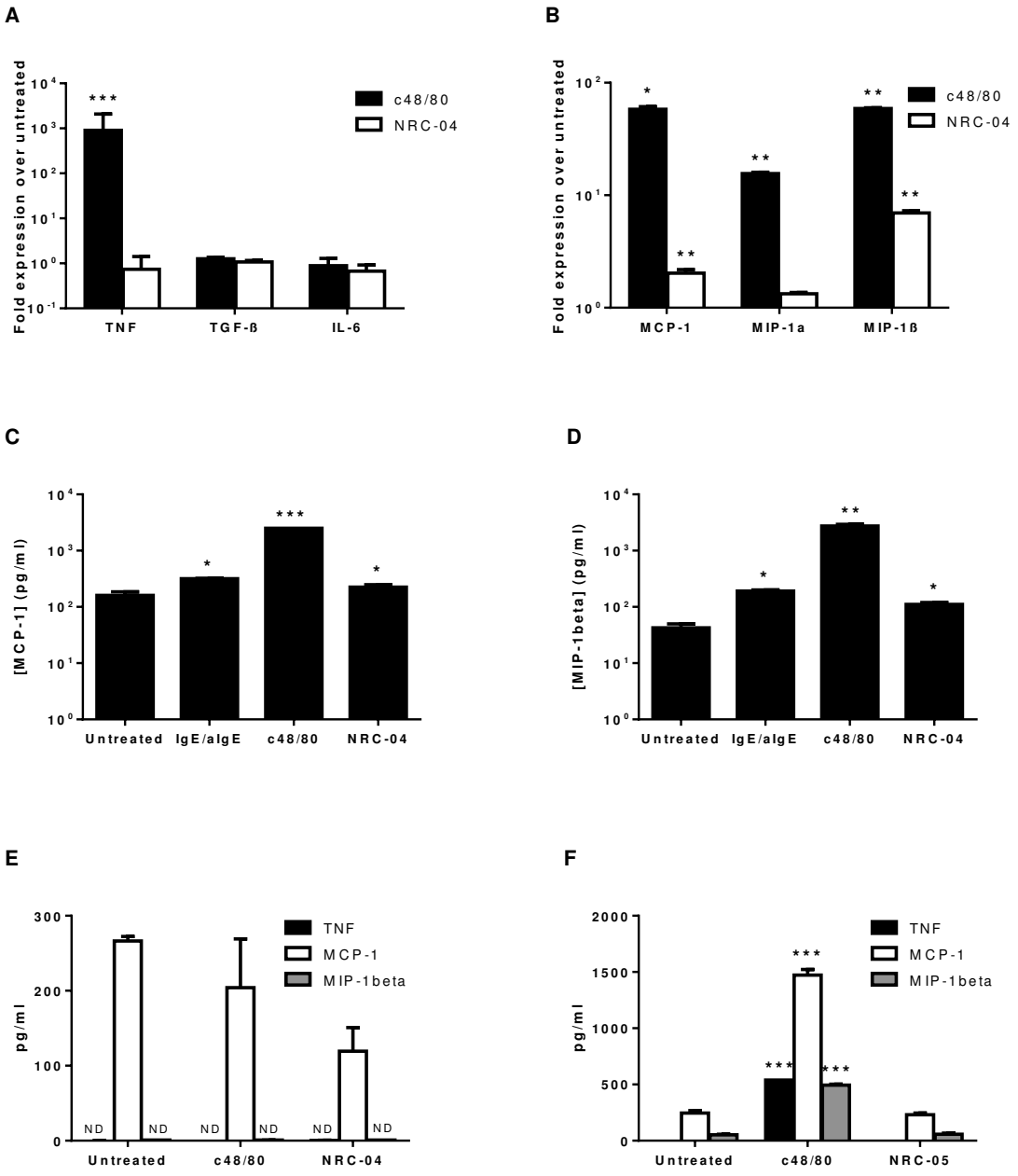

Figure 2.4 Pleurocidin NRC-04 induces the production and release of proinflammatory mediators. After 3 h treatment, mRNA expression of (**A**) TNF, TGF-β, and IL-6, (**B**) MCP-1, MIP-1α, and MIP-1β was measured by qPCR (n=3). Data was normalized to GAPDH mRNA levels and are expressed as fold increase over unstimulated controls. After 24 h treatment, production of (**C**) MCP-1, and (**D**) MIP-1β in cell-free supernatants was measured by ELISA (n=3). Production of TNF, MCP-1, and MIP-1β in cell-free supernatant from (**E)** c48/80 or NRC-04-treated HuMC and (**F**) c48/80 or NRC-05-treated LAD2 cells was measured by ELISA (n=3). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared with untreated. ND, not detectable.

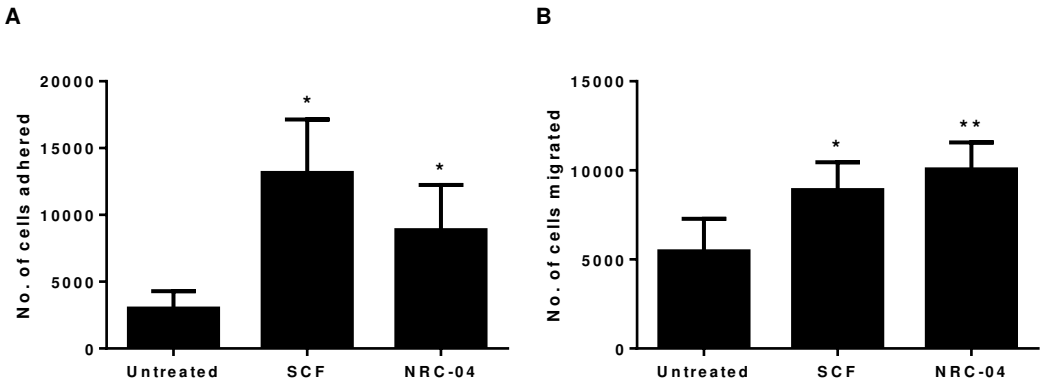

Figure 2.5 Pleurocidin NRC-04 mediates human mast cell adhesion and migration. (**A**) LAD2 cells were allowed to adhere for 2 h to fibronectin-coated wells in the presence of medium alone, 0.1 µg/ml SCF, or 1 µM NRC-04 and number of adherent cells was assessed as described in Methods section (n=3). (**B**) LAD2 cells were allowed to migrate for 6 h across inserts towards medium alone, 0.1 µg/ml SCF, or 1 µM NRC-04 and migration was assessed as described in Methods section (n=3). * $P<0.05$, ** $P<0.01$ compared with untreated.

2.4.7 Human mast cell activation by pleurocidin NRC-04 is sensitive to inhibitors of G protein signaling

Although the signaling pathways responsible for cathelicidin activation of mast cells are poorly understood, a number of specific secondary messengers such as G proteins and lipid kinases have been implicated (Chapter 1.5.1.1.5). We examined whether G protein, phosphoinositol 3-kinase (PI3K), phospholipase C (PLC) and/or protein kinase C (PKC) pathways were involved by pretreating the cells with pathway specific inhibitors, pertussis toxin, wortmannin, U-73122 and Ro-31-8220, respectively before activation with NRC-04. All four inhibitors significantly inhibited NRC-04-activated mast cell degranulation (**Figure 2.6A-D**), and pertussis toxin significantly suppressed NRC-04-induced intracellular Ca^{2+} mobilization in LAD2 cells (**Figure 2.6E, F**).

2.4.8 Human mast cells express N-formyl peptide receptor-like 1 (FPRL1) and $P2X_7$ receptors

Pertussis sensitivity suggested that NRC-04 was activating G protein signaling mediated by the activation of functional GPCR. Previous studies have shown that cathelicidin LL-37 activates mast cell chemotaxis via FPRL1 and mast cell cytokine release via purinoreceptor $P2X_7$. We hypothesized that FPRL1 and/or $P2X_7$ receptors might therefore be involved in NRC-04-mediated mast cell activation. RT-PCR analysis showed that LAD2 cells expressed mRNA for both FPRL1 and $P2X_7$ receptors (**Figure 2.7A**), and flow cytometric analysis confirmed cell surface expression of FPRL1 (**Figure 2.7B**).

To determine if NRC-04 activation was FPRL1 and $P2X_7$ dependent, we pretreated LAD2 cells with WRW4 (FPRL1 antagonist) or KN-62 ($P2X_7$ antagonist) prior to activation with NRC-04. WRW4 inhibited NRC-04-induced degranulation (**Figure 2.7C**), whereas KN-62 had no effect (**Figure 2.7D**).

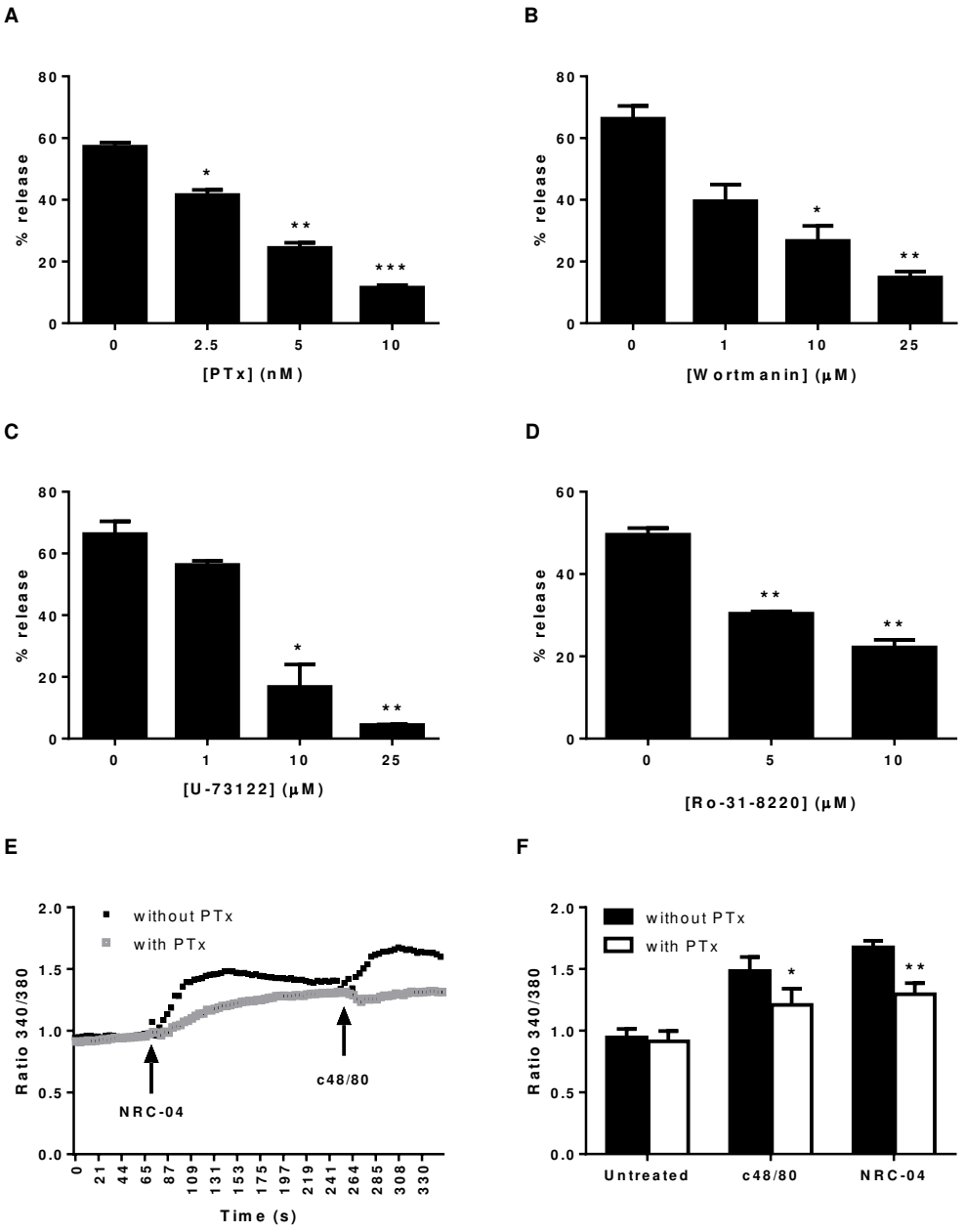

Figure 2.6 Pleurocidin NRC-04-induced human mast cell degranulation is G protein-, PI3K-, PLC- and PKC-dependent. LAD2 cells were treated with the indicated concentrations of (**A**) pertussis toxin (PTx), (**B**) wortmannin, (**C**) U-73122, or (**D**) Ro-31-8220 prior to stimulation with 1 μM NRC-04 and the percentages of β-hexosaminidase release were measured (n=3). (**E**) LAD2 cells were pretreated for 2 h with 5 nM PTx, loaded with fura-2, stimulated with 1 μM NRC-04 at time point 70 s followed with 0.5 μg/ml c48/80 at time point 256 s and mobilization of intracellular Ca^{2+} was measured. (**F**) The Ca^{2+} response in 20 randomly selected individual LAD2 cells reported as mean ± S.E.M. of ratio 340/380 (n=5). * P<0.05, ** P<0.01 compared with untreated.

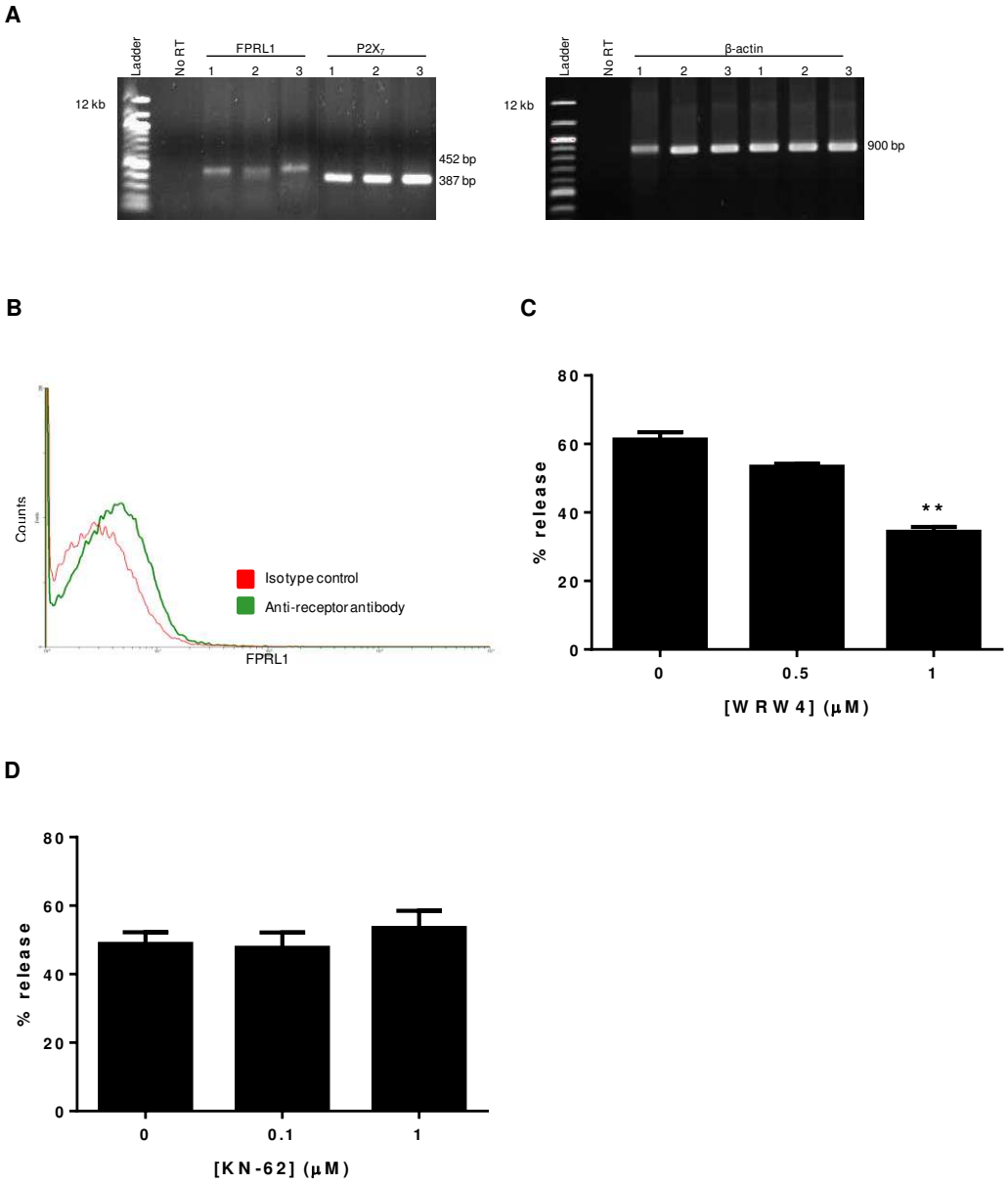

Figure 2.7 FPRL1 and $P2X_7$ receptors in NRC-04-mediated human mast cell activation. (**A**) Total RNA was isolated from LAD2 cells, and FPRL1 and $P2X_7$ receptor mRNA expression was examined by RT-PCR. β-actin was used as a positive control. (**B)** The expression of FPRL1 on LAD2 cell surface was evaluated by flow cytometry. LAD2 cells were pretreated for 2 h with (**C**) WRW4, (**D)** KN-62, or vehicle, stimulated with 1 μM NRC-04 and percentage of β-hexosaminidase release was measured (n=3). ** P<0.01 compared with untreated.

2.4.9 Fluorescence-resonance energy transfer (FRET)-based detection of FPRL1 activation by pleurocidin NRC-04

To determine whether NRC-04 could directly activate the FPRL1 receptor, we used a reporter assay in which FPRL1 is stably integrated into the Gα15-NFAT-*bla* CHO-K1 cell line. This cell line contains a beta-lactamase (*bla*) reporter gene under the control of a NFAT (nuclear factor of activated T-cell) response element and a promiscuous G protein, Gα15, stably integrated into CHO-K1 cells. In the absence of *bla* expression (unstimulated) cells appear green fluorescent (530 nm emission); however, in the presence of *bla* expression (stimulated), the FRET-enabled substrate is cleaved and cells appear blue fluorescent (460 nm emission). Incubation of cells with NRC-04 resulted in a significant increase in 460/530 (Blue/Green) emission ratio in a concentration-dependent manner. WKYMVM (W(m)) peptide was used a positive control. WRW4 and pertussis toxin were able to inhibit NRC-04 activity (**Figure 2.8**), indicating a specific interaction between NRC-04 and FPRL1.

2.5 DISCUSSION

In the present study, we investigated the pleurocidin family of CAPs in innate immune responses based on their abilities to activate human mast cells. We demonstrated that pleurocidin NRC-04 induced mast cell migration, adhesion, degranulation, release of lipid mediators, and the production of chemokines. By contrast, NRC-05 did not activate LAD2 cell degranulation and chemokine production, suggesting that these two functions are controlled by similar signaling pathways. Our data further showed that pleurocidins activated G proteins and PI3K, PLC and PKC signaling pathways in human mast cells. A specific GPCR, FPRL1, was expressed in human mast cells and likely to be functional in pleurocidin-induced mast cell activation.

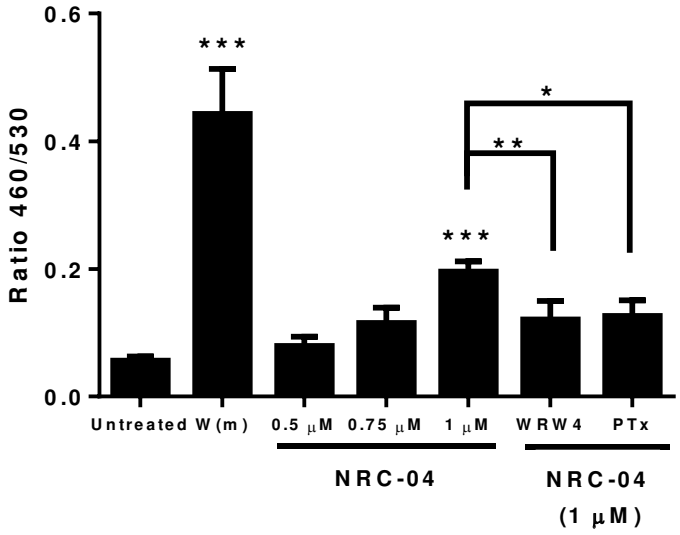

Figure 2.8 Pleurocidin NRC-04 couples to FPRL1 receptor. FPRL-1-Gα15-NFAT-*bla* CHO-K1 cells were incubated for 4 h with WRW4 or pertussis toxin (PTx), stimulated with W(m) or NRC-04 and FRET assay was performed as described in Methods section (n=3). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared with untreated.

Among the mucus-derived fish AMPs, the pleurocidin family is probably the best characterized and has been under investigation by several research groups. A study by Chiou *et al.* reported the potential immunoregulatory effects of pleurocidin on the expression of immune-relevant genes, IL-1 and cyclooxygenase (COX)-2, in a fish macrophage cell line (18), suggesting a proinflammatory role of pleurocidin in vertebrate immune system. In other studies, pleurocidin CAPs exhibited cytolytic activity against breast cancer cells and multiple myeloma cells and impaired the growth of tumor xenografts in immune-deficient mice (19), indicating that these peptides may also stimulate an antitumor immune response. Similar to cathelicidins, a family of mammalian CAPs, pleurocidin genes share highly conserved N-terminal signal peptide (*pre*) and C-terminal acid peptide (*pro*) sequences, in addition to the mature pleurocidin sequences (20-22), this results in an initial *pre*-pleurocidin-*pro* peptide, which is the precursor of mature and fully bioactive pleurocidin. Both mature cathelicidins (20) and mature pleurocidins form α-helixes and kill Gram-positive and Gram-negative bacteria by forming pores in their membranes (12, 23), causing cell lysis (11, 24). Different peptides belonging to the pleurocidin family are only found in pleuronectid flatfish (Winter flounder, Yellowtail flounder, American plaice, and Halibut) and share a common signal and proregion amino acid sequences but differ in core antimicrobial sequences (10). Pleurocidin family peptides exhibit a wide range of antimicrobial activities, ranging from highly potent (minimum inhibitory concentration < 1 μg/ml) to inactive. Peptides NRC-03, -04, -06, -07, -11, -12, -13, -15, -16, and -17, exhibit significant inhibitory activities against pathogens, and the peptide sequences of NRC-04, -07, -11, -12, and -13 CAPs consist of an XVGK motif, where X is H, K, or T, all of which are relatively bulky residues. In the case of NRC-04, the motif is even doubled as HVGKHVGK, which is probably compatible with a highly bactericidal amphipathic helix (10). Interestingly, the widely divergent antimicrobial activities of pleurocidins extend also to their ability to

activate mast cells. Our initial screen of the twenty pleurocidin peptides revealed that some peptides were capable of degranulating human mast cells. Eleven of the twenty pleurocidin peptides tested (NRC-02, -03, -04, -07, -08, -12, -13, -16, -17, -18, and -20) induced significant LAD2 degranulation. Pleurocidin NRC-04 showed the highest degranulation activity, and since it is also a potent CAP with maximum antimicrobial activity as compared with the other 19 peptides, it was used for subsequent studies.

A hallmark of mast cell activation is the rise in intracellular Ca^{2+} upon agonist stimulation and polybasic compounds such as c48/80 are potent activators of mast cell degranulation and calcium influx (25, Chapter 1.2.5.2). Intracellular Ca^{2+} is thought to play a key role in mast cell migration, release of histamine and lipid mediators (26). Ca^{2+} influx is required for histamine release by human β-defensin-2 and LL-37 (7). In this study, pleurocidin increased intracellular Ca^{2+} mobilization in mast cells, causing them to degranulate and release proinflammatory mediators.

De novo-synthesized lipid mediators are mainly secreted by activated mast cells, and are mediators of proinflammatory reactions (27, Chapter 1.2.6.2). We showed that pleurocidin activated the release of CysLTs and PGD_2 in human mast cells, in contrast with LL-37 which does not induce PGD_2 production (7). However, the study involving LL-37 used rat peritoneal mast cells; thus, differences in mast cell phenotypes should be considered. Our results with c48/80 were consistent with a previous report that in mast cells, c48/80 induced intracellular Ca^{2+} mobilization, high secretion of histamine and low release of PGD_2 (28). The failure of c48/80 to induce secretion of lipid mediators, suggests that, unlike c48/80, which activates human mast cells in a receptor-independent manner by directly acting on G proteins, pleurocidins utilize a receptor for their action.

Our findings demonstrated that pleurocidins activated the production of chemokines MCP-1 and MIP-1β, but not cytokines, implying that stimulation may be

selective for a limited number of proinflammatory chemokines. MCP-1 and MIP-1β are involved in recruitment and activation of various immune cells such as neutrophils, basophils, eosinophils, monocytes, and T cells to sites of tissue injury, infection, and inflammation (29, Chapter 1.2.6.3). Taken together, our results suggest that in addition to histamine and lipid mediator release, pleurocidins may also participate in the regulation of inflammatory immune response by the induction of chemokines and subsequent immune cell recruitment.

During inflammation, localized increases in the numbers of mast cells occurs, and their accumulation requires directed migration of these cells (1, 2, Chapter 1.2.7). The present study showed that pleurocidins can serve as a potent chemoattractant for human mast cells. Moreover, it promoted mast cell adhesion to fibronectin, indicating its importance in tissue localization of mast cells and a possible role in processes such as wound healing and fibrosis.

To understand the molecular mechanisms underlying the activities of pleurocidin, we investigated the requirement for G proteins, PI3K, PLC and PKC as their roles in CAPs-stimulated mast cell activation has been previously reported (4, Chapter 1.5.1.1.5). The G protein inhibitor pertussis toxin, the PI3K inhibitor wortmannin, the PLC inhibitor U-73122 and the PKC inhibitor Ro-31-8220 showed inhibitory effects on pleurocidin-mediated mast cell degranulation. In addition, pertussis toxin also inhibited the increase in intracellular Ca^{2+} mobilization. Cationic peptide stimulation of G proteins lead to the activation of PLC and PI3K; activated PLC catalyzes the generation of inositol triphosphate and diacylglycerol leading to Ca^{2+} mobilization and PKC activation respectively (25, Chapter 1.3, Figure 1.7). Among isoenzymes of PLC identified, only β subtype PLC is activated by G proteins, typically $G\alpha_q$. In addition, βγ subunits of G proteins which have dissociated from $G\alpha_{i/o}$ also regulate PLC β activity (30, 31); and since pertussis toxin is known to inhibit $G\alpha_{i/o}$ but not $G\alpha_q$ (32), our findings suggest that

$G\alpha_i$ and/or $G\alpha_o$ type G proteins are involved in pleurocidin-induced activation of PLC in mast cells. Thus, the activation of G protein-coupled PLC β pathway likely plays a role in the pleurocidin-elicited intracellular Ca^{2+} mobilization leading to mast cell activation. We also tested the effect of fluticasone propionate, salmeterol xinafoate, and tranilast on NRC-04-activated mast cell degranulation but found that none of these inhibitors had an effect on mast cell activation (data not shown).

Next we identified a functional receptor for pleurocidin in mast cells. LL-37 chemoattracts immune and inflammatory cells, including neutrophils, monocytes, and T cells via the action on a low affinity formyl peptide receptor, FPRL1 (33, Chapter 1.5.1.1). LL-37 also interacts with the purinoreceptor $P2X_7$, that is predominantly expressed on monocytes, macrophages and dendritic cells and induces the processing and release of the potent cytokine IL-1β (34) and IL-8 in human gingival fibroblasts (35). Thus, we addressed the question as to whether the pleurocidin-induced mast cell degranulation was mediated by FPRL1 and/or $P2X_7$**.** We found that both FPRL1 and $P2X_7$ were expressed by human mast cells. The FPRL1 antagonist WRW4 inhibited pleurocidin-induced mast cell degranulation but the $P2X_7$ antagonist KN-62 had no effect. Thus, pleurocidin utilizes FPRL1 as a receptor to activate mast cellss. In a fluorescent reporter assay utilizing FPRL1 stably integrated into the Gα15-NFAT-*bla* CHO-K1 cell line under the control of beta-lactamase reporter gene, we showed that pleurocidin activated a conformational change in FPRL1 which was sensitive to a pharmacological inhibitor of FPRL1 and pertussis toxin. FPRL1 belongs to the GPCR superfamily that activates host defense mechanisms in immune cells including migration, adhesion, and degranulation. It mediates these cellular functions in a pertussis toxin-sensitive manner, indicating coupling to one or more members of the $G\alpha_i$ subfamily of G proteins. The novel ability of FPRL1 to convey both pro and anti-inflammatory signaling makes it an intriguing, yet unusual GPCR (36). Not surprisingly numerous peptide

ligands of FPRL1 have been identified as potential therapeutic interventions for human disease (Chapter 1.5.1.1.4). Our study describes a novel, naturally-occurring proinflammatory agonist for FPRL1.

In conclusion, our studies show that pleurocidin could effectively recruit and activate human mast cells by binding and signaling through the $G\alpha_i$-coupled stimulatory FPRL1 receptor. Pleurocidin activation of mast cells not only induced degranulation and release of preformed granule mediators, but it also induced the production of chemokines. Pleurocidin-induced release of mast cell mediators could potentially lead to recruitment and activation of T lymphocytes, eosinophils, and other inflammatory cells that contribute to innate and adaptive immune responses. With the global emergence of many new infectious diseases, as well as concerns about the antibiotic resistance of an increasing number of microbial pathogens, the peptide antibiotics group of antimicrobials warrants attention and further studies. Our data may prove useful in suggesting novel ways of activating mast cells to boost immune responses.

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CHAPTER 3. THE COMPLEMENT COMPONENT C5a ACTIVATES HUMAN MAST CELLS VIA THE NOVEL C5a RECEPTOR, C5L2

A version of this chapter has been submitted for publication in The Journal of Immunology as follows:

P Pundir, C MacDonald, M Kulka. The novel receptor C5L2 is required for C5a-mediated human mast cell adhesion, migration, cytokine and chemokine production.[2]

3.1 ABSTRACT

Mast cells are tissue-resident immune effector cells that upon activation can initiate inflammatory responses to infectious insult or allergens by releasing a multitude of biologically-active mediators. The complement component C5a can regulate mast cell functions. However, the signaling pathways through which it acts remain unclear. C5a functions though two identified receptors C5a receptor (C5aR) and C5a receptor-like 2 (C5L2). C5aR is a classical G protein-coupled receptor known to mediate most mast cell responses associated with C5a, but the role of C5L2 has yet to be defined. We hypothesized that C5a activates human mast cells via the C5aR and C5L2 receptors. The human mast cell line, LAD2 (Laboratory of Allergic Diseases 2), expressed C5L2 receptor but not C5aR, whereas human peripheral blood $CD34^+$ cell-derived mast cells showed no surface expression of C5aR or C5L2. Although, C5a did not induce degranulation, it caused LAD2 to adhere, migrate, and release cytokines and chemokines. Silencing C5L2 using lentiviral short hairpin RNA rendered the cells unresponsive to C5a. Adhesion of mast cells could be blocked partially by pertussis toxin (G protein inhibitor) and wortmannin (phosphoinositol-3 kinase inhibitor); however, U-73122 (phospholipase C inhibitor) and Ro-31-8220 (protein kinase C) had no effect.

[2] P Pundir designed and performed the experiments, analyzed the data and wrote the chapter; M Kulka helped design the study and experiments, and edited the manuscript

Moreover, C5a caused extracellular signal-regulated kinases (ERK)-1/2 phosphorylation in LAD2 cells. Exposure to C5a caused ~60% internalization of cell surface C5L2 receptor. Culture in the presence of interleukin (IL)-4 or human fibroblasts led to upregulation of C5L2 receptor in human mast cells which correlated with enhanced adhesion and migration in response to C5a. These findings suggest that C5L2 receptor, which couples with pertussis toxin-sensitive signaling pathways, activates adhesion, migration and mediator release by human mast cells.

3.2 INTRODUCTION

Mast cells play a central role in the pathophysiology associated with asthma and allergic diseases (1, Chapter 1.2.10). The classical activation of mast cells occurs via the cell surface high affinity immunoglobulin (Ig)E receptor, FcεRI. Following cross-linking of FcεRI by allergens, mast cells are activated and secrete a multitude of biologically active mediators such as histamine, leukotrienes and prostaglandins, as well as proteases, cytokines and chemokines, which are all pivotal to the development of allergic inflammation (2, Chapter 1.2.5.1). Besides FcεRI, other IgE-independent mechanisms have been described for mast cell activation which can be initiated by a variety of peptides, secretagogues, and pathogen-associated molecular patterns (PAMPs) (2-4, Chapter 1.2.5.2, Figure 1.3)

The complement system is an evolutionarily conserved part of the innate immune system playing an important role in the host defense against pathogens (5, Chapter 1.5.2). After encountering PAMPs, activation of the complement system occurs through a proteolytic cascade. During the cascade numerous biologically active fragments are generated from zymogens, including the anaphylatoxic peptides C3a (6), C4a (7), and C5a (6, Figure 1.9). C5a causes very potent pathophysiologic responses, including smooth muscle contraction, enhanced vascular permeability (6, 8), and chemotaxis of macrophages, neutrophils, activated lymphocytes, basophils, and eosinophils (9-12, Chapter 1.5.2.2.4). C5a activates various immune cells through interaction with two different receptors, the C5a receptor (C5aR) and C5a receptor-like 2 (C5L2) (Chapter 1.5.2). C5aR and C5L2 are co-expressed on a wide variety of immune cells, including neutrophils, monocytes, mast cells, and immature dendritic cells and in tissues, such as testis, spleen, lung, liver, kidney, heart, and brain (13-16). C5aR is a classical G protein-coupled receptor (GPCR) through which most of the functional effects of C5a occur, such as chemotaxis, oxidative burst, degranulation, and release of proinflammatory

mediators (Chapter 1.5.2.2). C5L2 is also a 7-transmembrane protein that shares 58% sequence homology with the C5aR (13). It is a second high affinity receptor for C5a. which also binds C5a desArg with a much higher affinity than the C5aR (16). Some studies have also demonstrated that the C5L2 has the ability to bind C3a desArg (17-19). The functional role of C5L2 remains much more enigmatic, but some studies suggest that it may act as a non-signaling decoy receptor that regulates the bioavailability of C5a and thus limits C5aR-dependent cell activation. On the contrary, other studies suggest a functional role for C5L2 in controlling both proinflammatory and anti-inflammatory responses of C5a in different disease settings (17, Chapter 1.5.2.3).

Mast cells have long been considered a target for the action of anaphylatoxins and studies have shown that C5a induces mediator release by human mast cells (20), as well as chemotaxis of neoplastic human mast cells (HMC-1), cord blood-derived mast cells (CBMC) and skin mast cells (21, 22). In addition to chemotaxis and degranulation, C5a also induces cytokine and chemokine gene expression in mast cells (23, 24) and C5a-induced responses are inhibited by receptor-specific antibodies and pertussis toxin, an inhibitor of the $G\alpha_i$ family of G proteins. Rat basophilic-like (RBL-2H3) cells transfected with the C5aR release mediators in response to C5a stimulation (25). Therefore, the C5aR signaling cascade has been implicated in C5a-induced mast cell functions. However, a role for C5L2 has yet to be defined (Chapter 1.5.2.3). We hypothesized that C5a activates human mast cells through their C5aR and C5L2 receptors. The aims of our study were to determine: (i) the expression of C5aR and C5L2 by human mast cells, (ii) the ability of C5a in activating mast cells, (iii) whether C5a-mediated mast cell activation is C5aR- and C5L2-dependent, and (iv) the signaling pathway(s) used for the activation of mast cells by C5a. These studies addressed the overall hypothesis of this thesis which is that human mast cells express both stimulatory and inhibitory GPCR which signal through $G\alpha_i$ or $G\alpha_s$, respectively, and that these

receptors are important in chemotaxis and mast cell migration. We chose C5a receptors as our model of activating GPCRs. C5aR couples with stimulatory $G\alpha_i$; the expression, function and signaling mechanism of C5L2 in human mast cells are unknown.

3.3 METHODS

3.3.1 Cell culture

LAD2 (Laboratory of Allergic Diseases 2) human mast cell line was cultured in serum-free medium (StemPro-34 SFM, Life Technologies, Burlington, ON, Canada) supplemented with 2 mM $_L$-glutamine, 100 U/ml penicillin, 50 µg/ml streptomycin and /ml 0.1 µg/ml recombinant human stem cell factor (rhSCF; Peprotech, Rocky Hill, NJ, USA). The cells were maintained at a density of 0.1×10^6 cells/ml at 37°C/5% CO_2. The cells were periodically tested for expression of Kit and FcεRI by flow cytometry.

Human peripheral blood $CD34^+$ cells-derived mast cells were kindly provided by Dr. Dean Befus. These cells were cultured in StemSpan SFEM (StemCell Technologies, Vancouver, BC, Canada) supplemented with 2 mM $_L$-glutamine, 100 U/ml penicillin, 50 µg/ml streptomycin, 0.1 µg/ml rhSCF, and 0.1 µg/ml rh(interleukin) IL-6 (Peprotech); 0.03 µg/ml rhIL-3 (Peprotech) was added for the first week. At 8-10 weeks, the cultures consisted of >99% c-Kit^+ FcεRI^+ mast cells, as tested by flow cytometry.

The 1079SK human fibroblast cell line (ATCC CRL-2097) was cultured in Minimum Essential Medium (Life Technologies) supplemented with 2 mM $_L$-glutamine, 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 50 µg/ml streptomycin and 10% FBS.

3.3.2 Isolation of RNA and generation of cDNA by reverse transcription

Total RNA was isolated using the Tri Reagent method (Sigma-Aldrich Canada, Oakville, ON, Canada). In all, 1 µg of total cellular RNA was reverse-transcribed to cDNA

using M-MLV Reverse Transcriptase (Life Technologies) in a 20-µl reaction mix, according to the manufacturer's recommendation.

3.3.3 Reverse transcription PCR (RT-PCR)

RT-PCR was performed using a Peltier Thermal Cycler System (Bio-Rad, Hercules, CA, USA) with Life Technologies reagents. C5a receptor (C5aR), C5a receptor-like 2 (C5L2), and β-actin primers (**Table 3.1**) were designed using the Primer Express software (Applied Biosystems, Foster City, CA, USA). Amplification was carried out for 30 cycles including denaturation for 45 s at 94°C, annealing for 30 s at 52°C for C5aR, 58°C for C5L2, and 60°C for β-actin, and extension for 30 s at 72°C. PCR amplicons were analyzed by 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

3.3.4 Real-time qPCR

Gene expression was analyzed using real-time qPCR on a StepOnePlus system (Applied Biosystems). For each qPCR assay, a total of 50 ng of cDNA was used. Primer sets (**Table 3.1**) for PCR amplifications were designed using the Primer Express software. All reactions were performed in triplicate for 40 cycles as per the manufacturer's recommendation. All data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) internal controls (26) and are reported as fold change in expression over untreated cells.

3.3.5 Flow cytometric analysis

Cells were washed, suspended at 0.2×10^6 cells/ml in 0.1% bovine serum albumin (BSA)/PBS and/or permeabilized with 0.4% saponin and stained for 1 h with either phycoerythrin (PE)-labeled anti-C5aR antibody (R&D Systems, Minneapolis, MN, USA), PE-anti-C5L2 antibody (Abcam, Cambridge, MA, USA), or PE-isotype control antibody at

Table 3.1 Sequences of oligonucleotides used for PCR

Gene	Forward primer	Reverse primer	Probe: FAM/TAMRA (GAPDH: MAX/BHQ)
GAPDH	5'-TCG TGG AAG GAC TCA TGA C-3'	5'-CCA TCA CGC CAC AGT TT-3'	5'-/5MAXN/AGT CCA TGC CAT CAC TGC CAC/3IABlk_FQ/-3'
C5aR	5'-TGA CTG AAG AGT CCG TGG TTA GG -3'	5'-CTG GGT CTT CTG GGC CAT AG-3'	5'-/56-FAM/ AGA GCA AGT CAT TCA CGC GCT CCA C/36-TAMSp/-3'
C5L2	5'-CCA GGG CCA GGA CGA AA-3'	5'-CTC CAG CCT ACA CCT CCA TCT C-3'	5'-/56-FAM/ TGG ACA GCA AGA AAT CCA CCA GCC A/36-TAMSp/-3'
GM-CSF	5'-CAG CCC TGG GAG CAT GTG-3'	5'-ATT CAT CTC AGC AGC AGT GTC TCT A-3'	5'-/56-FAM/AGG CCC GGC GTC TCC TGA ACC/36-TAMSp/-3'
IL-1β	5'-TGA GCT CGC CAG TGA AAT GA-3'	5'-TGA GCT CGC CAG TGA AAT GA-3'	5'-/56-FAM/GAA GCT GAT GGC CCT AAA CAG ATG AAG TGC T/36-TAMSp/-3'
IL-6	5'-AGC CAC TCA CCT CTT CAG AAC GAA-3'	5'-AGT GCC TCT TTG CTG CTT TCA CAC-3'	5'-/56-FAM/ ACG GCA TCT CAG CCC TGA GAA AGG AGA /36-TAMSp/-3'
IL-8	5'-CTG GCC GTG GCT CTC TTG-3'	5'-TTG GCA AAA CTG TTT AGC ACT CC-3'	5'-/56-FAM/ CAG CCT TCC TGA TTT CTG CAG CTC TGT GT /36-TAMSp/-3'
IP-10	5'-CGA TTC TGA TTT GCT GCC TTA TC-3'	5'-TGA TTA CTA ATG CTG ATG CAG GTA CA-3'	5'-/56-FAM/TGG CAT TCA AGG AGT ACC TCT CTC TAG AAC CGT /36-TAMSp/-3'
MIP-1β	5'-CAG CGC TCT CAG CAC CAA-3'	5'-TTC CTC GCG GTG TAA GAA AAG-3'	5'-/56-FAM/ CTC AGA CCC TCC CAC CGC CTG C/36-TAMSp/-3'
MCP-1	5'-TCT CTG CCG CCC TTC TGT -3'	5'- GCC TCT GCA CTG AGA TCT TCC T-3'	5'-/56-FAM/ CTG CTC ATA GCA GCC ACC TTC ATT CCC /36TAMSp/-3'
RANTES	5'-TCG CTG TCA TCC TCA TTG CTA-3'	5'-GCA CTT GCC ACT GGT GTA GAA A-3'	5'-/56-FAM/ CTG GGA CAC CAC ACC CTG CTG C /36-TAMSp/-3'
TGF-β	5'-CTC TCC GAC CTG CCA CAG A-3'	5'-AAC CTA GAT GGG CGC GAT CT-3'	5'-/56-FAM/ CCC TAT TCA AGA CCA CCC ACC TTC TGG T /36-TAMSp/-3'
TNF	5'-TCT GGC CCA GGC AGT CA-3'	5'-GCT TGA GGG TTT GCT ACA ACA TG-3'	5'-/56-FAM/ CAG TGC TCC AAG CCC GGT GTC ATC /36TAMSp/-3'
C5aR	5'-ATG GAT CCT AAC ATA CCT GCG GAT G-3'	5'-TCT ACA CCG CCT GAC TCT TCC G-3'	
C5L2	5'-AAC CAC ACC ACC AGC GAG TAT TAT G-3'	5'-AGC CCT CTT GCC TAC ACC GGC-3'	
β-actin	5'-ATC TGG ACC ACA CCT TCT ACA ATG AGC TGC G-3'	5'-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC-3'	

C5aR, C5a receptor; C5L2, C5a receptor-like 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GM-CSF, granulocyte macrophage colony-stimulating factor; , IL, interleukin; IP-10, inducible protein-10; MIP, monocyte inflammatory protein; MCP-1, monocyte chemotactic protein-1; RANTES; regulated upon activation, normal T cell expressed and secreted; TGF-β, transformation growth factor-beta; TNF, tumor necrosis factor.

4℃. Cells were washed twice, resuspended in 0.1% BSA/PBS and analyzed on a FACSArray or FACSCalibur (BD Biosciences, Mississauga, ON, Canada).

3.3.6 Degranulation assay

Cells were washed, resuspended in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.38 mM $Na_2HPO_4.7H_2O$, 5.6 mM glucose, 1.8 mM $CaCl_2.H_2O$, 1.3 mM $MgSO_4.7H_2O$, 0.4% BSA, pH 7.4) at 2.5×10^4 cells per well and stimulated for 30 min with serial dilutions of C3a (Calbiochem, Billerica, MA, USA) or C5a (Biovision, Milpitas, CA, USA) at 37℃/5% CO_2. β-hexosaminidase released into the supernatants and in total cell lysates solubilized with 0.1% Triton X-100 was quantified by hydrolysis of p-nitrophenyl N-acetyl-β-D-glucosamide (Sigma-Aldrich) in 0.1 M sodium citrate buffer (pH 4.5) for 90 min at 37℃/5% CO_2. The percentage of β-hexosaminidase release was calculated as a percent of total content.

In some experiments, LAD2 cells were sensitized overnight with 0.5 μg/ml biotinylated immunoglobulin E (IgE, Abbiotec. San Diego, CA, USA). Cells were washed with HEPES, preincubated for 30 min with C5a, stimulated for 30 min with streptavidin (Life Technologies) and then β-hexosaminidase release was measured. In other experiments, LAD2 cells were treated for 30 min with C5a followed by stimulation for 30 min with compound 48/80 (c48/80; Sigma-Aldrich) or SCF and β-hexosaminidase release was measured.

3.3.7 Intracellular Ca^{2+} mobilization

LAD2 cells were loaded for 30 min with 1 μM fura-2 AM (Life Technologies) in HEPES buffer, washed and incubated for 15 min in BSA-free HEPES at 37℃/5% CO_2. A total of 4×10^6 cells were placed in glass-bottom culture dish under an inverted microscope (Axiovert 200, Carl Zeiss Canada Ltd., Canada). Fura-2 was excited at 340

and 380 nm alternatively. The Ca^{2+} response was recorded at 100 ms intervals using SlideBook for Stallion, version 4.26.04 software (Intelligent Imaging Innovations, USA). In all, 0.1 µg/ml C5a was added at the 70 s time point and 1 µM ionomycin (Sigma-Aldrich) at the 256 s time point. The Ca^{2+} response of 20 randomly selected cells were analyzed for each experiment and plotted as the ratio 340/380 vs. time.

3.3.8 ELISA

1×10^6 cells were stimulated for 3 h either with 0.5 µg/ml IgE/100 µg/ml anti-IgE or 0.1 µg/ml C5a at 37°C/5% CO_2. Cell-free supernatants were isolated and analyzed for cysteinyl leukotrienes (CysLTs), prostaglandin D_2 (PGD_2) or cytokine content using the following commercial competitive enzyme immunoassay (EIA) kits: correlate-EIA cysteinyl leukotriene kit (Assay Designs, Ann Arbor, MI, USA), prostaglandin D_2 EIA kit (Cayman Chemicals, Ann Arbor, MI, USA), human tumor necrosis factor (TNF)-alpha quantikine ELISA kit, and human granulocyte-macrophage colony-stimulating factor (GM-CSF) quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA). The minimum detection limits were CysLT, 78.1 pg/ml; PGD_2, 200 pg/ml; TNF, 5.5 pg/ml; and GM-CSF, 3 pg/ml.

3.3.9 Cytometric bead array

0.3×10^6 cells were stimulated for 24 h with 0.0001-0.1 µg/ml C5a at 37°C/5% CO_2. Cell-free supernatants were isolated and analyzed for chemokine content using the human chemokine cytometric bead array kit (BD Biosciences). The minimum detection levels are inducible protein-10/CXCL10 (IP-10), 2.9 pg/ml; monocyte chemoattractant protein-1/CCL2 (MCP-1), 2.7 pg/ml; monokine induced by interferon-γ/CXCL9 (MIG), 2.5 pg/ml; RANTES/CCL5 (regulated on activation, normal T cell expressed and secreted), 1 pg/ml; and IL-8, 0.2 pg/ml.

3.3.10 Adhesion assay

Maxisorp 96-well plates (NUNC, Naperville, IL, USA) were coated for 16 h with 10 µg/ml human fibronectin (Sigma-Aldrich) in PBS at 4°C, washed three times with PBS, blocked for 1 h with 3% BSA in HEPES buffer at 37°C/5% CO_2 and then washed three times with HEPES buffer. Cells were washed with HEPES buffer, suspended at 1 × 10^6 cells/ml, and labeled for 20 min with 5 µM calcein-AM (Life Technologies) at 37°C/5% CO_2. After labeling, cells were washed and resuspended at 1 × 10^6 cells/ml in HEPES buffer. Cell suspension (5 × 10^4 cells) ± HEPES buffer containing 0.1 µg/ml SCF or 0.01-1 µg/ml C5a was added and incubated for 2 h at 37°C/5% CO_2. In some experiments, cells were pretreated for 30 min with 10 nM wortmannin (Sigma-Aldrich), 10 nM U-73122 (Calbiochem), 10 nM Ro-31-8220 (Sigma-Aldrich) or vehicle, or for 2 h with 5-10 nM pertussis toxin (Sigma-Aldrich). After incubation, non-adherent cells were washed away with warm HEPES buffer. Fluorescence emission at 530 nm (485 nm excitation) was measured using a fluorescence plate reader (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, Nepean, ON, Canada).

3.3.11 Chemotaxis assay

Chemotaxis was performed using a 96-well monocyte cell migration kit (Calbiochem) with slight modifications. Briefly, cells were incubated overnight in SCF-free media, washed with HEPES buffer, and suspended at 0.25 × 10^6 cells/ml. Agonists (0.1 µg/ml SCF or 0.01-1 µg/ml C5a) were added to the lower chamber of the 96-well tray and cell suspension (2.5 × 10^4 cells) was added to the insert (upper chamber). The cells were allowed to migrate towards the agonists for 6 h at 37°C/5% CO_2. After incubation, the insert was washed twice with warm HEPES buffer. Cells were labeled for 30 min with 5 µM calcein-AM at 37°C/5% CO_2. After labeling, the cells that had migrated

across the insert membrane were lysed using 0.1% Triton X-100. Fluorescence emission was measured at 530 nm (485 nm excitation).

3.3.12 Lentivirus-mediated knockdown of C5L2

C5L2 small hairpin RNA (shRNA) lentiviral particles and scrambled control shRNA lentiviral particles (Santa Cruz Biotechnology, Dallas, TX, USA), which do not bind to any known human mRNAs, were transduced into LAD2 cells according to the manufacturer's protocol. Briefly, 2×10^6 LAD2 cells were infected by adding 40 µl shRNA lentiviral particles (multiplicity of infection 1) in the presence of 2 µg/ml polybrene (Santa Cruz Biotechnology). cop-green fluorescent protein (GFP) control lentiviral particles were used to confirm the transduction of LAD2 cells. At 72 h post-infection, the medium was changed to virus-free complete medium, and antibiotic selection (2 µg/ml puromycin, Santa Cruz Biotechnology) was initiated 7 days later. Cells were analyzed for C5L2 knockdown by flow cytometry and used for subsequent assays following initiation of puromycin selection.

3.3.13 Receptor internalization

Cells (0.25×10^6) were exposed to 0.1% BSA/PBS buffer or 0.1 µg/ml C5a for 30 min at 37℃, washed twice with ice-cold 0.1% BSA/PBS buffer, stained with PE-anti-C5L2 antibody or PE-isotype control antibody and incubated on ice for 1 h. After washing twice with cold 0.1% BSA/PBS, the cells were fixed in a 2% formaldehyde solution and analyzed using flow cytometry. The percentage of receptor internalization was calculated from the mean fluorescent intensity as follows:

(MFI[exp.]-MFI[0% control])/(MFI[100% control]-MFI[0% control]) *100

MFI: mean fluorescent intensity; 100% control corresponds to C5L2 expression on the cell surface of untreated cells; 0% control refers to cells incubated with isotype control antibody.

3.3.14 Western blot

Mast cells (1×10^6) were stimulated with 0.1 µg/ml C5a for 0, 1, 5, 10, 20, or 30 min and were lysed in buffer containing loading dye solution (lithium dodecyl sulfate) sample buffer (Life Technologies), 5% β-mercaptoethanol (Sigma-Aldrich), 5 mM dithiothreitol (Sigma-Aldrich) and protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Whole cell lysates were separated on 4-12% Bis-Tris SDS-PAGE gels (Life Technologies) and transferred onto nitrocellulose membranes. The membranes were blocked with 4% BSA in Tris-buffered saline (TBS)-0.05% Tween overnight and then probed with primary antibodies against phospho-ERK1/2 (Thr202/Tyr204; Cell Signaling Technology Inc.) in 4% BSA-TBS-Tween for 1 h at room temperature. The membranes were washed with TBS-Tween four times and then incubated with the horseradish peroxidase-linked secondary antibody (donkey anti-mouse IgG-HRP; R&D Systems) for 1 h. The membrane was stripped and reprobed with β-actin antibody. The membranes were developed with chemiluminescence reagent (Life Technologies) for 1 min and exposed to chemiluminescence film for 1 to 5 min.

3.3.15 Co-culture of mast cells with fibroblasts

The 1079SK fibroblasts were seeded in 6-well tissue culture plates at 5000 cells per well. After 7 days, the medium was replaced with StemPro-34SFM containing 3×10^4 LAD2 cells per ml. The cells were incubated for 7 days after which the culture was trypsinized with 0.025% trypsin-EDTA to detach LAD2 cells from fibroblasts. Detached cells were incubated for 30 min in T75 flasks to remove any contaminating fibroblasts as only the fibroblasts were attached to the bottom, and most of the mast cells were recovered from the supernatants. The non-adherent mast cells were harvested, characterized for cell surface Kit expression by flow cytometry and used in subsequent studies.

3.3.16 Statistical analysis

Each experiment was performed at least three separate times. Statistical significance was determined by two-tailed Student's paired *t*-test and $P<0.05$ was considered significant. The results are shown as mean ± S.E.M.

3.4 RESULTS

3.4.1 Characterization of C5aR and C5L2 receptors in human mast cells

C5aR and C5L2 mRNA are abundantly expressed in several tissues and in peripheral blood granulocytes (13). We used qPCR to assess the expression of C5aR and C5L2 mRNA by LAD2 cells. The LAD2 human mast cell line was originally obtained from a patient with mastocytoma and the cells have many features common with primary $CD34^+$ cells-derived human mast cells. As shown in **Figure 3.1A**, LAD2 cells expressed C5aR and C5L2 mRNA. We next assessed the protein expression of these receptors using flow cytometry. Interestingly, no C5aR was detected on the cell surface. LAD2 cells expressed greater C5L2 receptor on their surface (**Figure 3.1B**). As shown in **Figure 3.1C**, the mean fluorescence intensity (MFI) of anti-C5L2 antibody-stained LAD2 cells was significantly higher (MFI 829 ± 3.6) compared with the cells stained with isotype control antibody (MFI 52 ± 5.5) or anti-C5aR antibody (MFI 53.3 ± 1.3). Since we could not find surface C5aR expression, we hypothesized perhaps C5aR was expressed internally in intracellular vesicles. Therefore, we determined the intracellular expression of C5aR and C5L2 by permeabilizing LAD2 cells with saponin and performing flow cytometry using antibodies specific to each receptor. Our data showed that LAD2 cells displayed a weaker expression of C5aR intracellularly in contrast to abundant expression of C5L2 (**Figure 3.1D**).

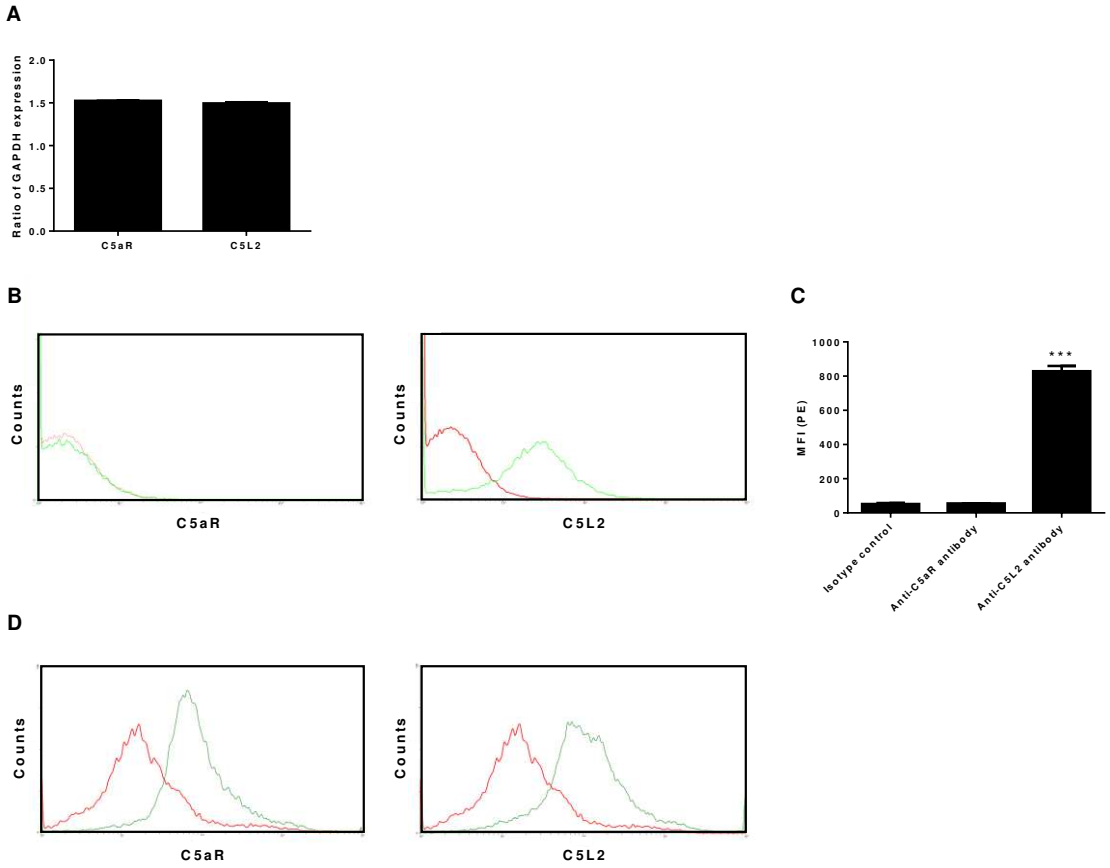

Figure 3.1 Expression of C5a receptors by human mast cells. (**A**) Total RNA was isolated from LAD2 cells, and expression of C5aR and C5L2 mRNA was examined by qPCR. Data was normalized to GAPDH mRNA levels and are expressed as fold expression over GAPDH expression. (**B**) The surface expression of C5aR and C5L2 by LAD2 was measured using flow cytometry and a phycoerythrin (PE)-labeled mouse IgG isotype control (red histogram), PE-anti-C5aR (green histogram), or PE-anti-C5L2 antibody (green histogram). (**C**) Summary of flow cytometric analysis expressed as differences in MFI between LAD2 cells stained with PE-isotype control, PE-anti-C5aR, or PE-anti-C5L2 antibody (n=3). (**D**) Intracellular expression of C5aR and C5L2 was analyzed in permeabilized LAD2 cells using flow cytometry. *** $P<0.001$ compared with isotype control.

Since LAD2 are sometimes considered to possess a neoplastic phenotype, we determined the expression of C5aR and C5L2 by human peripheral blood $CD34^+$ cells-derived mast cells (HuMC), which resemble the connective tissue-type mast cell phenotype, are considered to have a more mature mast cell phenotype and are often used as a model of skin mast cells. HuMC were identified by the expression of Kit receptor. Similar to LAD2 cells, HuMC did not express C5aR on their surface (**Figure 3.2A**). However, they expressed C5aR and significant amounts of C5L2 which were mostly located within intracellular compartments (**Figure 3.2B**).

To make sure that the anti-C5aR and anti-C5L2 antibodies that we used for our flow cytometry analysis were capable of recognizing C5aR and C5L2 on human mast cells, we tested HMC-1 cells, a mast cell line known to express C5aR and C5L2, for their expression of both receptors. As expected, our antibodies detected expression of both C5aR and C5L2 on the surface HMC-1 cells (**Figure 3.2C**). Based on the overall results, we focused on $C5aR^-$ $C5L2^+$ LAD2 cells for subsequent analyses.

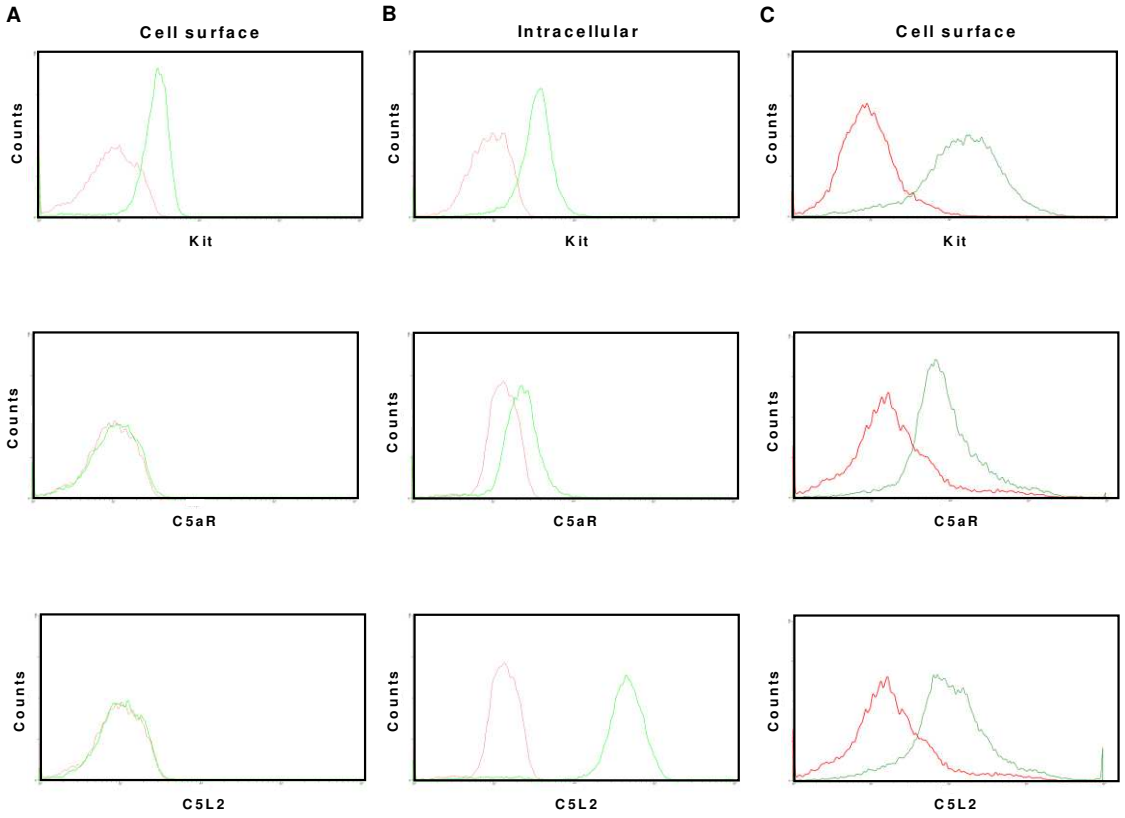

Figure 3.2 C5a receptors expression by HuMC and HMC-1 cells. (**A**) Cell surface and (**B**) intracellular expression of Kit, C5aR and C5L2 by HuMC and (**C**) cell surface expression of Kit, C5aR and C5L2 by HMC-1 cells was measured using flow cytometry and a phycoerythrin (PE)-labeled mouse IgG isotype control (red histogram), PE-anti-Kit, PE-anti-C5aR, or PE-anti-C5L2 antibody (green histograms).

3.4.2 Effects of interferon (IFN)γ and interleukin (IL)-4 on C5a receptors expression

Although some mast cells do not constitutively express C5aR, it is possible to induce its expression with certain stimuli. For example, mouse bone marrow-derived mast cells (BMMC) neither express cell surface C5aR nor respond to C5a, but preincubation of these cells with antigen, ionomycin, or phorbol 12-myristate 13-acetate (PMA) upregulates surface C5aR and makes BMMC responsive to C5a, inducing Ca^{2+} flux and chemotaxis (27). We thus assessed if incubation of mast cell with cytokines would modulate C5aR and C5L2 expression. First, we treated LAD2 cells with different concentrations of IFNγ for varying time points. IFNγ is a Th_1 cytokine that generally inhibits FcεRI-mediated mast cell activation. qPCR analysis showed that culturing LAD2 cells in different concentrations of IFNγ for 9 h upregulated the expression of C5aR (**Figure 3.3A**), however there was no significant effect on the expression of C5L2 at any of the concentrations and time points tested (**Figure 3.3B**). Then, we incubated LAD2 cells in different concentrations of IL-4 for similar time periods. IL-4 is a Th_2 cytokine which has been shown to potentiates FcεRI-mediated mast cell activation (29). Culture in the presence of IL-4 for 3-9 h increased mast cell expression of C5L2 mRNA after which the expression returned to basal level (**Figure 3.3D**). The expression of C5aR remained unchanged (**Figure 3.3C)**. Flow cytometric analysis confirmed that preincubation with IL-4 increased cell surface expression of C5L2 in a concentration-dependent manner (**Figure 3.3E)**.

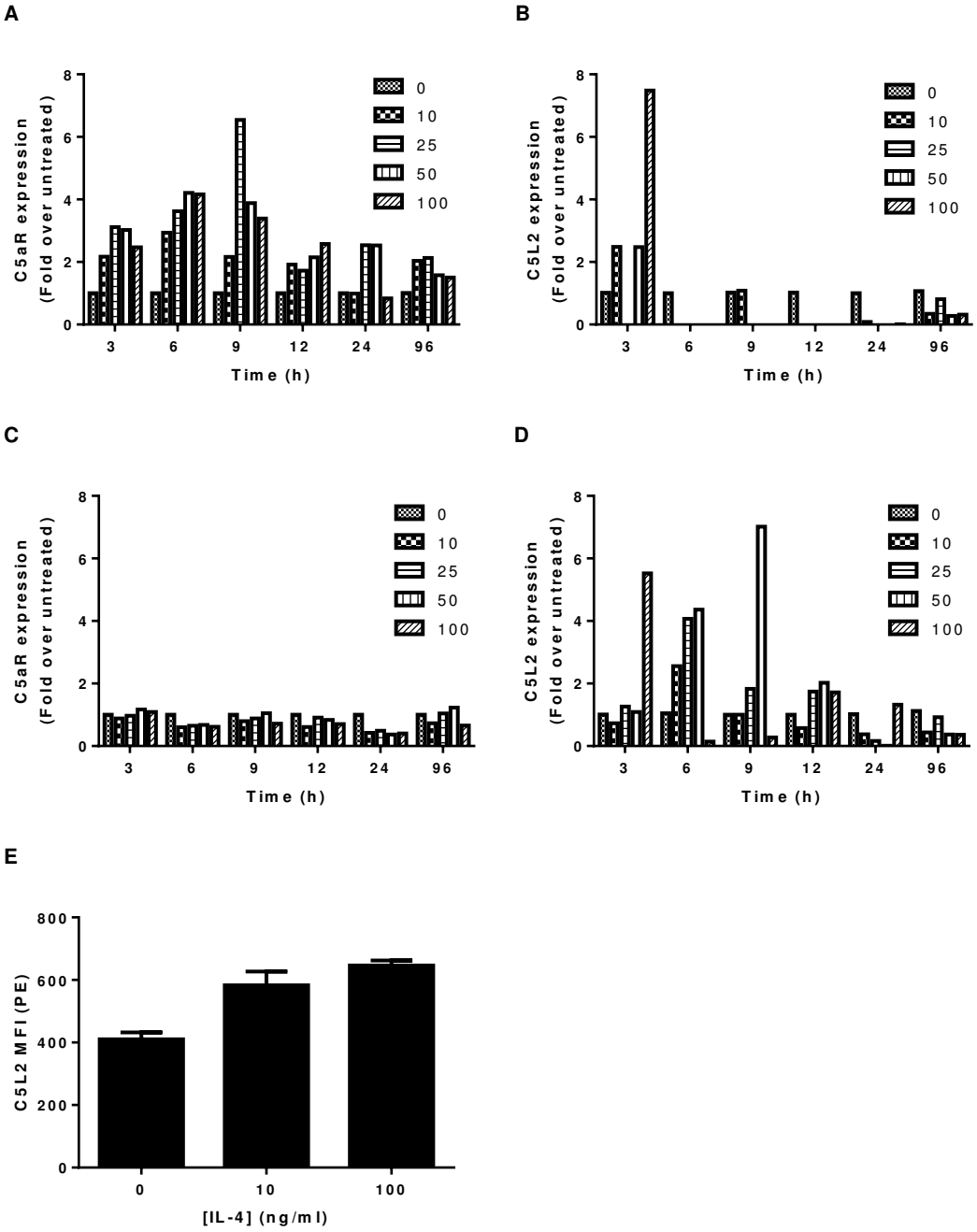

Figure 3.3 Effect of Th_1 and Th_2 cytokines on C5aR and C5L2 expression. LAD2 cells were cultured with (**A, B**) IFNγ or (**C, D)** IL-4 for 3, 6, 9, 12, 24, and 96 h and (**A, C**) C5aR or (**B, D)** C5L2 expression was measured by qPCR and (**E**) flow cytometry (n=3) ** P<0.01. *** P<0.001 compared with untreated.

3.4.3 Effect of C5a on mast cell degranulation and intracellular Ca^{2+} mobilization

Previous studies have shown that anaphylatoxins may contribute to hypersensitivity reactions by degranulating eosinophils, basophils as well as mast cells, but the studies with C5a on human mast cells have led to variable and inconclusive data. For instance, C5a has been found to degranulate skin mast cells but not lung and tonsillar mast cells. Some authors have reported that C5a degranulates LAD2 cells (30), while others have shown no effect (24). We compared the ability of C5a and C3a (another complement component established as a human mast cell secretogogue; Chapter 1.5.2.3) to degranulate human mast cells. LAD2 cells were treated with various concentrations of C3a and C5a, and degranulation was assessed by the release of β-hexosaminidase enzyme. As shown in **Figure 3.4A**, C5a was unable to stimulate LAD2 cell degranulation at any of the concentrations tested. C3a degranulated LAD2 cells in a concentration-dependent manner, with maximum degranulation (47.95 ± 1.65%) at 0.1 µg/ml.

As we demonstrated that LAD2 cells express higher C5L2 in the presence of IL-4, we thus determined the functional consequences of C5L2 upregulation. LAD2 cells were incubated in the absence or presence of 100 ng/ml IL-4 for up to 4 days and degranulation assay was performed with C5a. c48/80 was used as a positive control. Similar to IL-4-negative LAD2 cells, while IL-4-positive mast cells degranulated in response to c48/80, C5a failed to stimulate degranulation (**Figure 3.4B**).

Integrated signaling pathways have been suggested for mast cell activation. Mast cell stimulation with C3a or adensoine potentiates FcεRI-mediated degranulation. We thus determined if concurrent stimulation of mast cells via the FcεRI-, Kit- or G protein-signaling pathways would modulate C5a signal transduction. For the first set of experiments, we sensitized LAD2 cells overnight with 0.5 µg/ml biotinylated IgE prior to stimulation for 30 min with 0.5 µg/ml streptavidin and 0.1 µg/ml C5a, and determined the

release of β-hexosaminidase enzyme. As shown in **Figure 3.4C**, while IgE alone did not induced degranulation, treatment with IgE-streptavidin induced 32.7 ± 0.9% release of β-hexosaminidase. Stimulation with C5a alone or in combination with IgE and IgE-streptavidin did not potentiate degranulation of mast cells. We then determined the effect of simultaneous activation of G protein on C5a function by utilizing c48/80. We treated LAD2 with c48/80 in the absence or presence of 0.1 µg/ml C5a and measured degranulation. 0.5 µg/ml c48/80 activated 51.6 ± 0.8% release of total β-hexosaminidase content, whereas C5a had not effect on c48/80-induced mast cell degranulation (**Figure 3.4D**). Finally, we assessed the effect of Kit stimulation. We starved LAD2 cells of SCF by culturing them overnight in SCF-free medium followed by treatment with different concentrations of SCF in the absence or presence of 0.1 µg/ml C5a. As expected, stimulation with SCF failed to induce degranulation of mast cells. C5a was also unable to elicit degranulation in SCF-negative as well as SCF-positive mast cells (**Figure 3.4E**).

An increase in intracellular Ca^{2+} is necessary for the induction of mast cell degranulation, and C5a has been shown to mobilize intracellular Ca^{2+} to mediate its biological responses in various immune cells. As C5a failed to degranulate LAD2 cells, we hypothesized that C5a would not mobilize Ca^{2+} in LAD2 cells. Cells were loaded with the Ca^{2+}-sensing fluorescent dye fura-2 AM and treated with C5a, and changes in fluorescence were measured as a change in ratio of A_{340} to A_{380}. C5a did not cause an increase in intracellular Ca^{2+} concentration. As expected, stimulation with ionomycin (positive control) led to a transient increase in intracellular Ca^{2+} (**Figure 3.4F**).

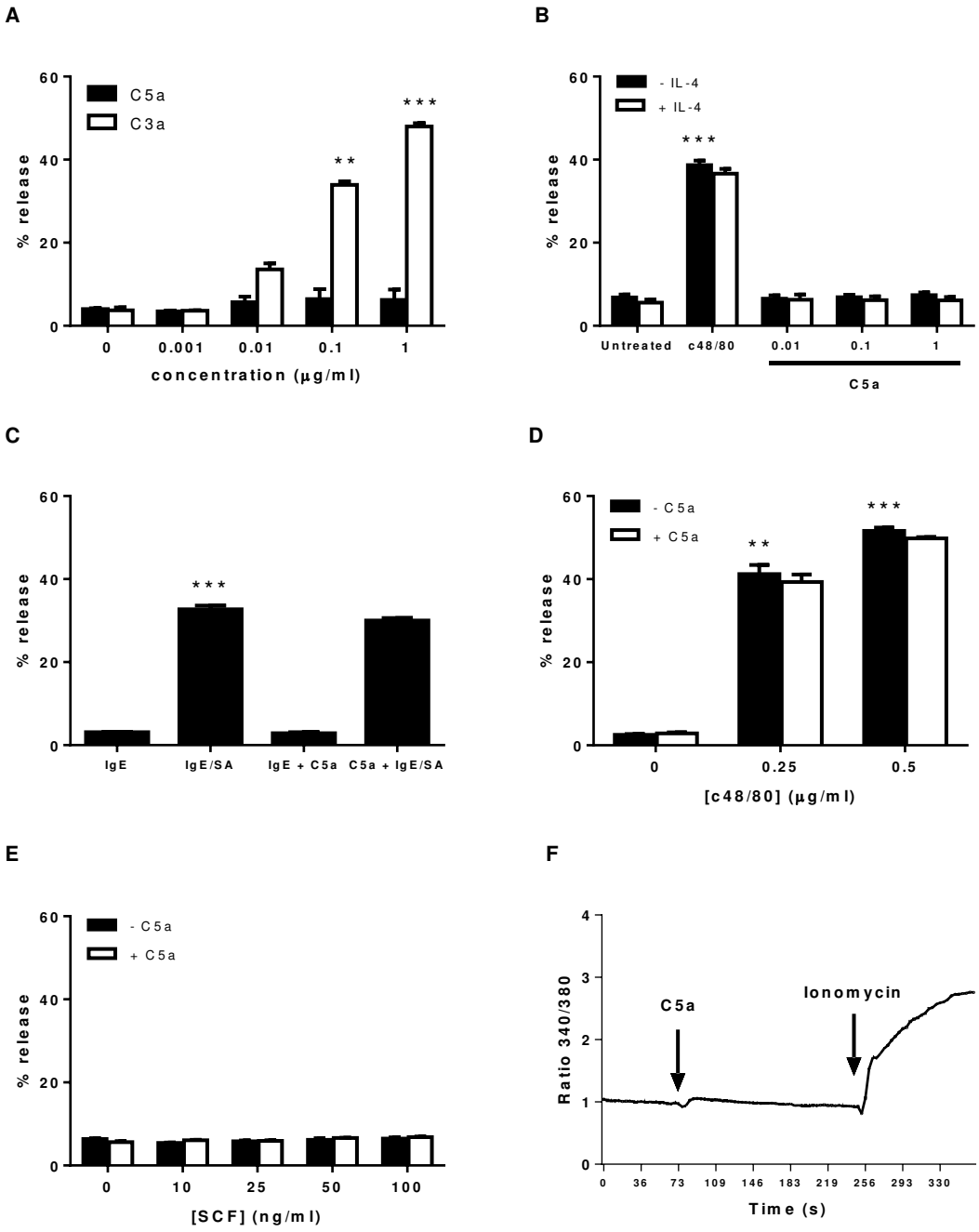

Figure 3.4 C5a does not induce mast cell degranulation. (**A**) LAD2 cells were stimulated for 30 min with C5a or C3a and β-hexosaminidase release was measured (n=3). (**B**) LAD2 cells were incubated for 4 days with 100 ng/ml IL-4, stimulated for 30 min with C5a and β-hexosaminidase release was measured (n=5). LAD2 cells were either sensitized with 0.5 µg/ml biotinylated-IgE or incubated in SCF-free media overnight, treated for 30 min with 0.1 µg/ml C5a followed by stimulation with (**C**) streptavidin (SA, 0.5 µg/ml), (**D**) c48/80, or (**E**) SCF for 30 min and β-hexosaminidase release was measured (n=3). Fura-2 loaded LAD2 cells were stimulated with 0.1 µg/ml C5a at time point 70 s followed with 1 µM ionomycin at time point 256 s and mobilization of intracellular Ca^{2+} was measured (n=3). ** P<0.01, *** P<0.001.

3.4.4 C5a stimulates production of cytokines and chemokines

Mast cell-derived cytokines and chemokines propagate a proinflammatory reaction. We therefore measured the expression of cytokines and chemokines after 3 h of C5a stimulation using qPCR. mRNA expression of GM-CSF, TNF (**Figure 3.5A**), IP-10/CXCL10, and MCP-1/CCL2 (**Figure 3.5B**) was modestly increased by C5a stimulation. Protein production of these mediators following 24 h stimulation with C5a was confirmed using cytometric bead array. C5a induced production of GM-CSF (15.1 ± 0.4 pg/ml per 300,000 cells) (**Figure 3.5C**), TNF (23.17 ± 1.8 pg/ml) (**Figure 3.5D**), IP-10 (32.5 ± 2.3 pg/ml) (**Figure 3.5E**), and MCP-1 (53.4 ± 4.5 pg/ml) (**Figure 3.5F**).

3.4.5 C5a induces adhesion, chemotaxis and upregulation of CD29 expression by human mast cells

Mast cell accumulation is critical in various pathophysiological conditions, including allergic reactions, atopic diseases, wound healing, tissue repair, and host defense against infection. Since C5a activates chemotaxis of HMC-1, CBMC and skin mast cells, we evaluated whether C5a would also promote adhesion and chemotaxis of LAD2 cells. Comparable to SCF, C5a activated LAD2 cells to adhere to fibronectin, an extracellular matrix protein, with maximum adhesion (366.4 ± 60%) at 0.01 µg/ml concentration (**Figure 3.6A**). As adhesion is closely related to cytoskeletal reorganization and migration, we measured LAD2 chemotaxis to either SCF or C5a. Similar to SCF, C5a activated LAD2 chemotaxis with maximum chemotaxis (143.1 ± 8%) at 1 µg/ml concentration (**Figure 3.6B**).

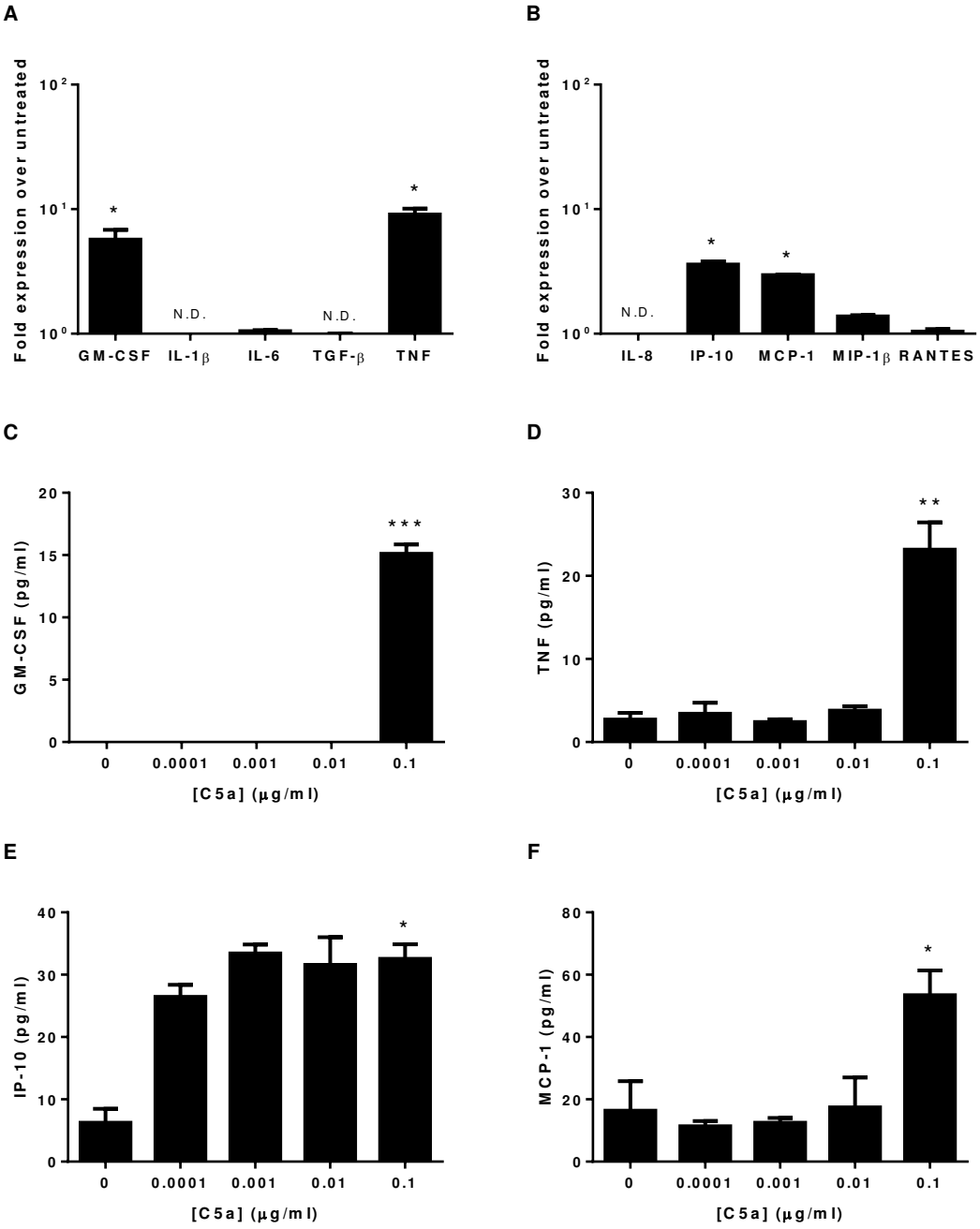

Figure 3.5 C5a induces the production of cytokines and chemokines. After a 3 h treatment with 0.1 µg/ml C5a, mRNA expression of (**A**) GM-CSF, IL-1β, IL-6, TGF-β, TNF, (**B**) IL-8, IP-10, MCP-1, MIP-1β, and RANTES was measured by qPCR (n=3). Data was normalized to GAPDH mRNA levels and are expressed as fold increase over untreated controls. After 24 h treatment of LAD2 cells (0.3 x 10^6 cells) with 0.0001 – 0.1 µg/ml C5a, production of (**C**) GM-CSF, (**D**) TNF, (**E**) IP-10 and (**F**) MCP-1 in cell-free supernatants was measured by cytometric bead array (n=5). * P<0.05, ** P<0.01, *** P<0.001 compared with untreated.

CD29 (β1 integrin) is the fibronectin receptor involved in a variety of cell-cell and cell-matrix interactions. It is broadly expressed on hematopoietic cells including mast cells, and is responsible for mast cell accumulation and function in inflamed tissues. As C5a stimulated LAD2 cell adhesion to fibronectin, we determined whether the effect was CD29-dependent. We treated LAD2 cells for 2 h with 0.1 μg/ml C5a and measured cell-surface CD29 expression using flow cytometric analysis. As shown in **Figure 3.6C**, the MFI of C5a-treated anti-CD29 antibody stained LAD2 cells was significantly higher (MFI 910 ± 31) than the untreated cells stained with either an anti-CD29 antibody or an isotype control antibody (MFI 650 ± 13).

3.4.6 C5L2 expression is required for C5a-mediated induction of mast cell adhesion, migration, and cytokine/chemokine production

To confirm whether C5a was stimulating mast cell responses by acting through the C5L2 receptor, we utilized small hairpin RNA (shRNA) lentivirus system to stably knockdown the expression of C5L2 in LAD2 cells. We transduced cells with shRNA lentiviral particles targeting mRNA of the C5L2 gene (LAD2-C5L2kd), and as a negative control, we used scrambled shRNA lentiviral particles (LAD2-cntr). To confirm transduction of LAD2 cells and determine transduction efficiency we employed copGFP control lentiviral particles. **Figure 3.7A** (right panel) shows data in which LAD2 cells were stably transduced with copGFP lentiviral particles at multiplicity of infection (MOI) 1. After transduction and selection with puromycin, flow cytometry was performed to evaluate C5L2 receptor knockdown. As shown in **Figure 3.7B**, we were able to generate LAD2 cells with 80% knockdown of the C5L2 expression. The MFI of anti-C5L2 antibody-stained LAD2-C5L2kd cells was significantly lower (MFI 191.3 ± 33.9) compared with that of LAD2-cntr (MFI 1395.3 ± 179) (**Figure 3.7C**). We then compared the cellular phenotype of LAD2- C5L2 with LAD2-cntr by testing these cells for Kit and

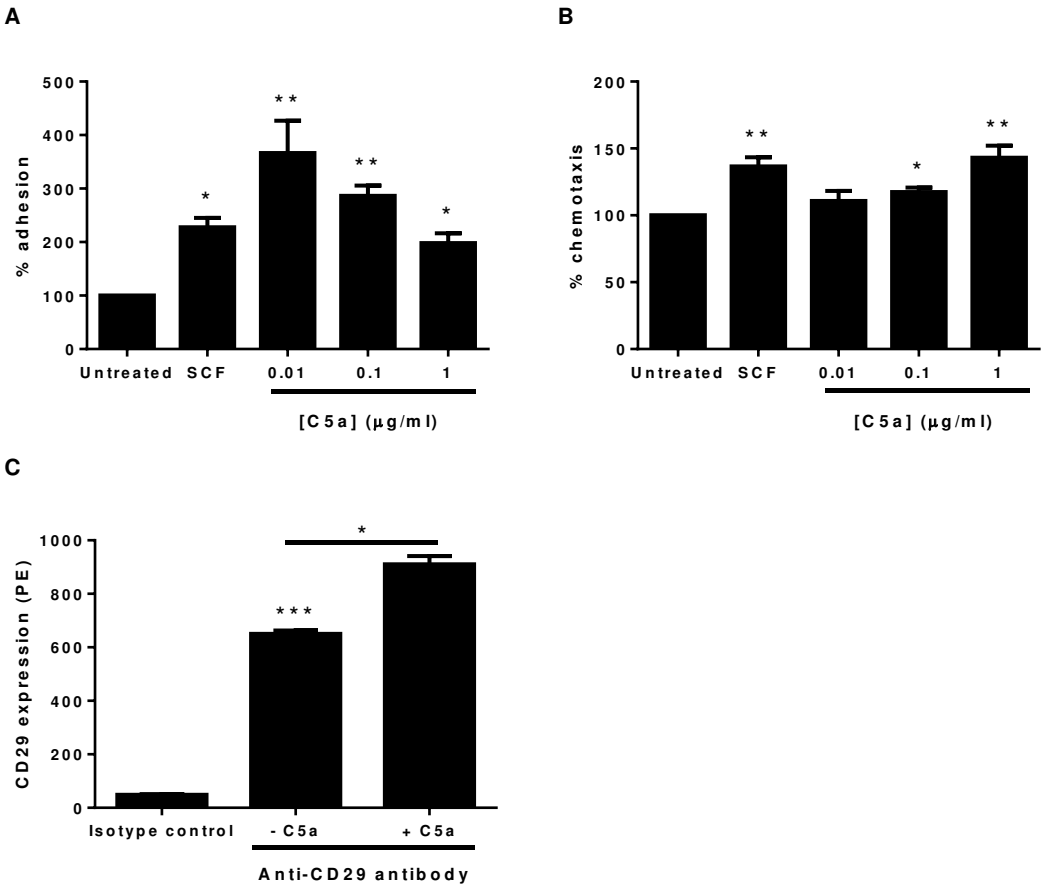

Figure 3.6 C5a mediates human mast cell adhesion, chemotaxis and CD29 upregulation. (**A**) LAD2 cells were allowed to adhere for 2 h to fibronectin-coated wells in the presence of medium alone, 0.1 µg/ml SCF, or 0.01-1 µg/ml C5a and percentage of cell adhesion over untreated cells was assessed (n=5). (**B**) LAD2 cells were allowed to migrate for 6 h across inserts towards medium alone, 0.1 µg/ml SCF, or 0.01-1 µg/ml C5a and percentage of cell chemotaxis over untreated cells was assessed (n=5). (**C**) LAD2 mast cells were treated with 0.1 µg/ml C5a for 2 h, fixed in 2% paraformaldehyde, incubated with PE-anti-C5L2 antibody or an isotype control and analyzed by flow cytometry (n=3). * $P<0.05$ compared to untreated. * $P<0.05$, ** $P<0.01$ compared with untreated.

FcεRI expression. There was no difference in expression of Kit and FcεRI between LAD2-C5L2kd and LAD2-cntr. Similarly, there was no significant difference in β-hexosaminidase release in response to c48/80 or C5a (data not shown).

We then examined if LAD2-C5L2kd cells would express cytokines and chemokine after C5a stimulation. LAD2-cntr and LAD2-C5L2kd cells were exposed to C5a and qPCR was performed. In LAD2-cntr cells, C5a caused a significant increase in GM-CSF and MCP-1 expression. In contrast, when LAD2-C5L2kd cells were stimulated with C5a, there was no expression of GM-CSF and MCP-1 (**Figure 3.8A, B**).

We further determined if C5a-induced mast cell adhesion was C5L2-dependent by allowing LAD2-cntr and LAD2-C5L2kd cells to adhere to fibronectin in the presence of C5a or SCF (positive control). When LAD2-C5L2kd cells were exposed to C5a, no adhesion was observed, whereas LAD2-cntr cells adhered to fibronectin (**Figure 3.8C**). Similarly, to see whether absence of C5L2 would affect C5a-induced mast cell chemotaxis, LAD2-cntr and LAD2-C5L2kd cells were allowed to migrate towards C5a or SCF (positive control). LAD2-cntr cells exhibited chemotaxis towards C5a, whereas LAD2-C5L2kd cells showed no chemotaxis towards C5a (**Figure 3.8D**). Collectively, these findings confirmed the role of C5L2 receptor as a mediator of C5a function in human mast cells.

3.4.7 C5a stimulation of human mast cells mediates C5L2 internalization, G protein and PI3K activation, and ERK1/2 phosphorylation

Agonist-induced activation of most GPCRs is followed by their desensitization and internalization (29). To determine whether C5L2 is internalized following C5a exposure, we incubated LAD2 cells for 30 min with C5a and measured the cell surface C5L2 expression by flow cytometry. Up to 60% of C5L2 was internalized within 15 min following exposure to C5a (**Figure 3.9A**).

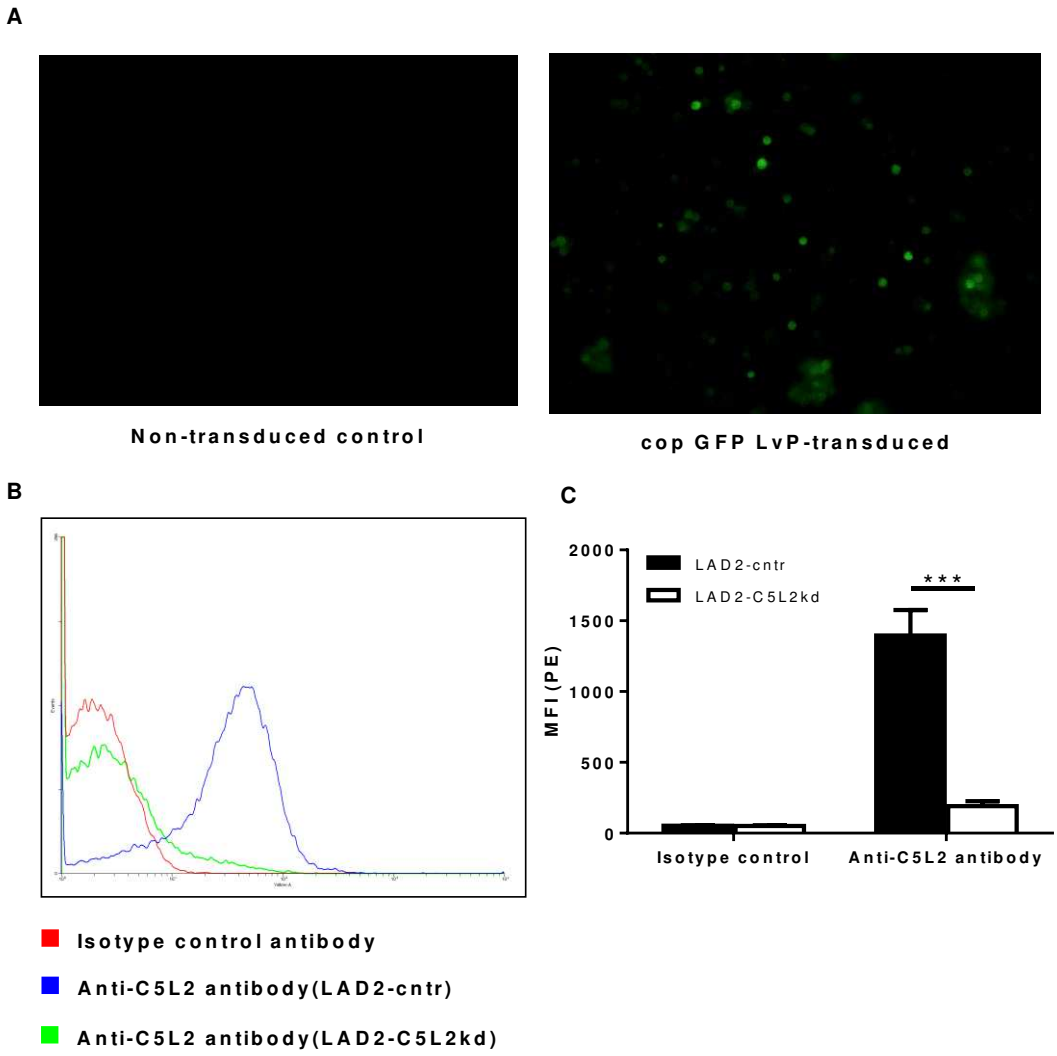

Figure 3.7 Stable knockdown of C5L2 in human mast cells. LAD2 cells were transduced with copGFP control lentiviral particles (LvP), scrambled shRNA control LvP or shRNA LvP targeted against C5L2. (**A**) LAD2 cells stably transduced with copGFP LvP (right) compared with non-transduced negative control (left). (**B**) Flow cytometry was performed to assess C5L2 expression levels on control (LAD2-cntr) and C5L2 shRNA LvP-transduced knockdown (LAD2-C5L2kd) cells. (**C**) Data is represented as MFI ± S.E.M. from three independent experiments. *** $P<0.001$.

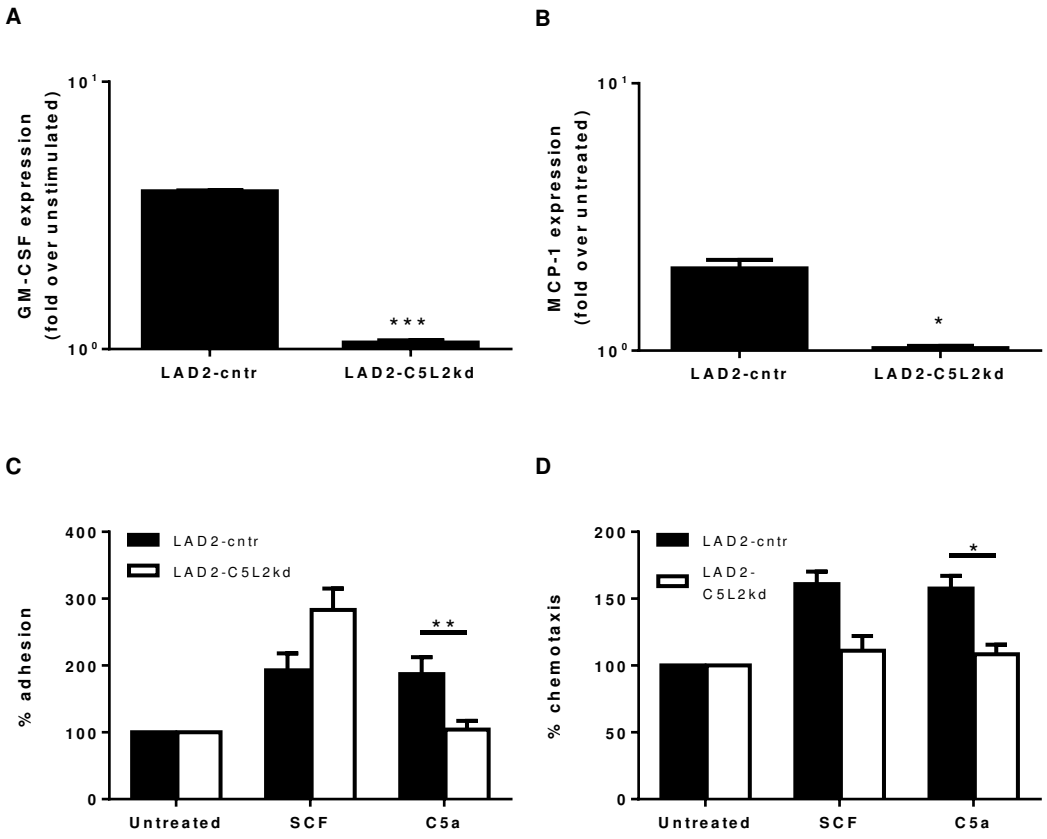

Figure 3.8 Effects of C5L2 knockdown on C5a-induced responses in human mast cells. Control (LAD2-cntr) and C5L2-knockdown (LAD2-C5L2kd) LAD2 cells were stimulated with 0.1 µg/ml C5a. After 3 h treatment, expression of (**A**) GM-CSF and (**B**) MCP-1 was measured by qPCR (n=5). (**C**) LAD2-cntr and LAD2-C5L2kd cells were allowed to adhere to fibronectin for 2 h in the presence of 0.1 µg/ml SCF or 0.1 µg/ml C5a and the percentages of cell adhesion were determined (n=5). (**D**) LAD2-cntr and LAD2-C5L2kd cells were allowed to migrate for 6 h towards 0.1 µg/ml SCF or 0.1 µg/ml C5a and the percentages of cell chemotaxis were determined (n=5). * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Although the functional role of C5L2 in human mast cells remains unknown, studies in other cell types have shown that C5L2 may weakly couple to the $G\alpha_i$ subunit, which is pertussis toxin-sensitive. We reasoned that the moderate response of C5a coupling to C5L2 in LAD2 cells could be due to G protein-dependent signal transduction. We tested our reasoning by performing an adhesion assay by treating cells with pertussis toxin prior to stimulation with C5a and measured the adhesion to fibronectin. As shown in **Figure 3.9B**, pretreatment with pertussis toxin inhibited C5a-induced adhesion of LAD2 cells to fibronectin. The inhibition, however, was not 100%, indicating that other signaling mechanism might be activated in C5a-mediated LAD2 cell adhesion.

As sensitivity to pertussis toxin indicated involvement of G protein signaling, we next determined if phosphoinositol 3-kinase (PI3K), phospholipase C (PLC) and/or protein kinase C (PKC) signaling molecules were involved in C5a-mediated mast cell adhesion. For these experiments, we treated the cells with wortmannin (PI3K inhibitor), U-73122 (PLC inhibitor) and Ro-31-8220 (PKC inhibitor) prior to stimulation with C5a and measured the adhesion to fibronectin. We found that wortmannin inhibited C5a-induced mast cell adhesion. U-73122 and Ro-31-8220 were ineffective at blocking C5a-mediated adhesion (**Figure 3.9C**).

The biological effects of many GPCRs are mediated via the activation of extracellular signal regulated kinase (ERK) signaling pathway. We performed western blot analysis to study tyrosine phosphorylation of ERK1/2 in LAD2 cells in response to C5a. C5a caused ERK1/2 phosphorylation within 1 min of stimulation that remained elevated for 5 min and was substantially eliminated at 10 min (**Figure 3.9D**).

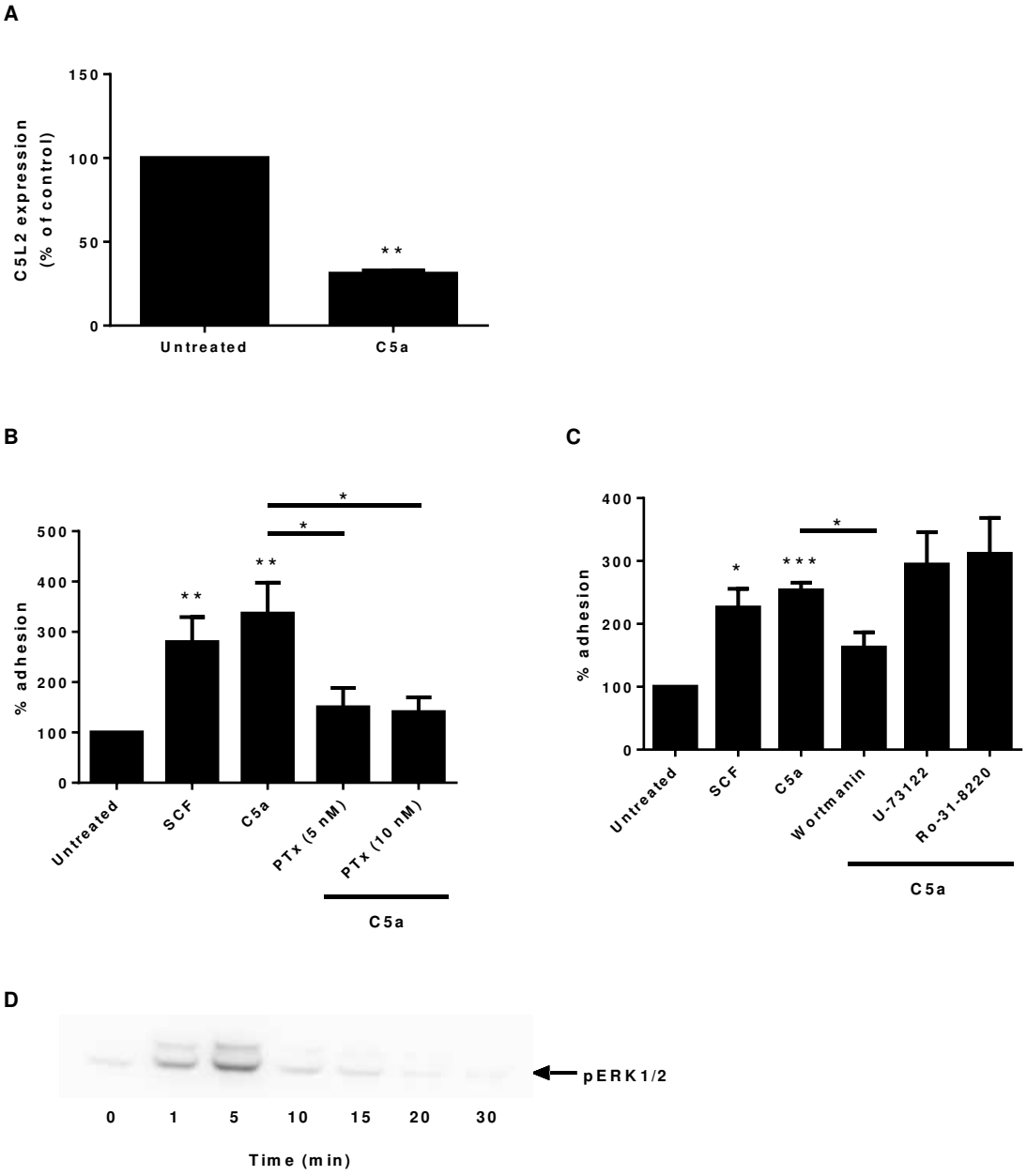

Figure 3.9 C5a induces C5L2 internalization, G protein and PI3K activation, and ERK1/2 phosphorylation. (**A**) LAD2 cells were exposed to 0.1 µg/ml C5a for 10 min, fixed in 2% paraformaldehyde, incubated with anti-C5aR antibody, anti-C5L2 antibody, or an isotype control antibody and analyzed by flow cytometry (n=3). (**B-C**) LAD2 cells were treated for 2 h with the indicated concentrations of PTx or for 30 min with wortmannin, U-73122, or Ro-31-8220 (10 nM each) prior to stimulation for 2 h with 0.1 µg/ml C5a and the percentages of cell adhesion were determined. 0.1 µg/ml SCF was used as a positive control (n=3). (**D**) LAD2 cells were stimulated with 0.1 µg/ml C5a for 0, 1, 5, 10, 15, 20 or 30 min and protein lysates were analyzed for phospho-ERK1/2 expression by western blot (n=3). * P<0.05, ** P<0.01, *** P<0.001 compared to untreated.

3.4.8 Co-culture of human mast cells with fibroblasts upregulates C5L2 and potentiates C5a-mediated adhesion and chemotaxis

Interaction of mast cells with fibroblasts leads to differentiation, maturation and enhanced mediator release (31-33). We questioned the effect of such interactions on mast cell responses to C5a. To address this, we established a culture system in which LAD2 cells were co-cultured with human fibroblasts 1079SK for a week in the presence of SCF. We examined C5a receptor expression in co-cultured LAD2 cells and found enhanced expression of C5L2. The MFI of anti-C5L2 antibody-stained co-cultured LAD2 cells was significantly higher (410.3 ± 20) compared with MFI of cells that were not cultured in the presence of 109SK (268.3 ± 16) (**Figure 3.10A**). No expression of C5aR was detected in LAD2 cells as well as co-cultured LAD2 cells (data not shown). We next investigated the response of co-cultured LAD2 cells to C5a. We found that C5a amplified mast cell adhesion (**Figure 3.10B**) and chemotaxis (**Figure 3.10C**). These results suggested that C5a-C5L2 signaling may aggravate mast cell responses depending upon their location and interactions with other structural and immune cells.

3.5 DISCUSSION

The studies reported here represent the first characterization of the novel receptor for C5a, C5L2, which is a functional receptor in human mast cells. C5a is one of the most powerful inflammatory mediators of the innate immune system, with the ability to initiate multiple cellular responses such as recruitment of inflammatory cells to sites of infection, activation of phagocytic cells and release of granule-associated mediators (6). Mast cells show highly variable responsiveness to C5a; skin human mast cells are activated by C5a (34), whereas lung (35) and intestinal mast cells (36) do not respond. Moreover, within the heterogenous lung mast cell population, there are two distinct subpopulations; M_{TC} that degranulate in response to C5a and M_T, that do not (37). The

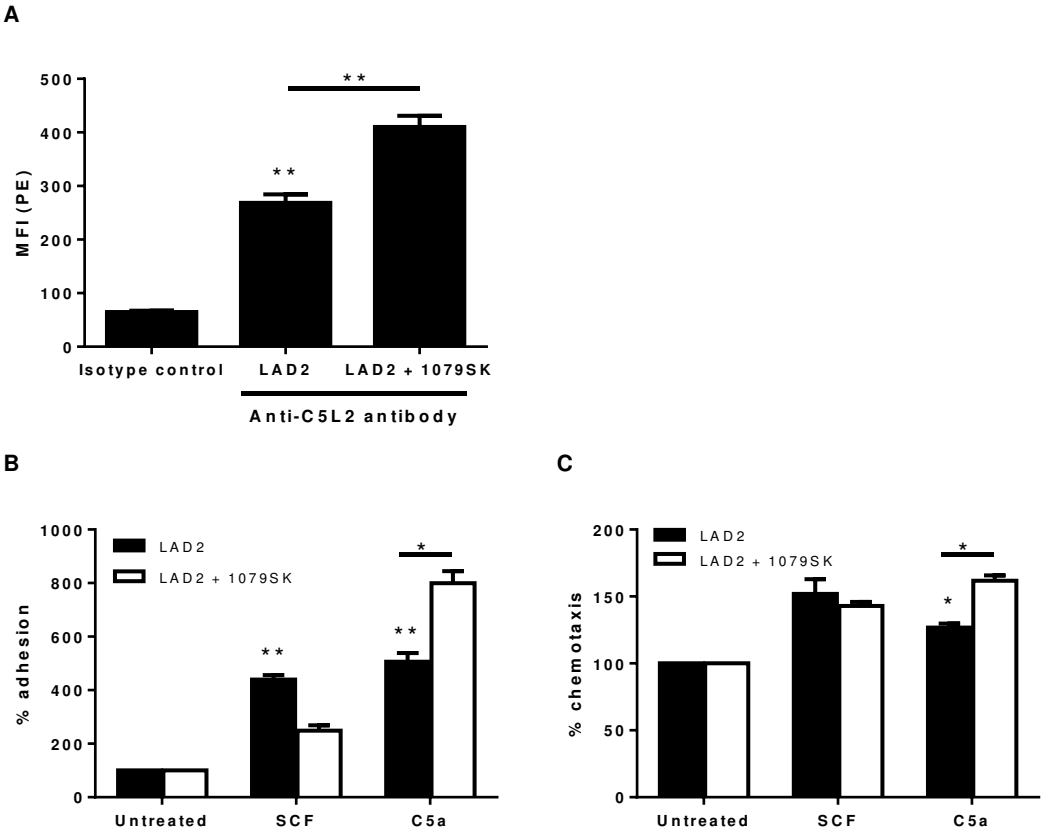

Figure 3.10 C5L2 expression, adhesion and chemotaxis by human mast cells cultured with fibroblasts. LAD2 cells were cultured with or without 1079SK fibroblasts for 7 days. (**A**) Cells were incubated with PE-anti-C5L2 antibody or an PE-isotype control antibody and C5L2 expression was analyzed by flow cytometry (n=3). (**B**) Cells were allowed to adhere to fibronectin for 2 h in the presence of 0.1 µg/ml SCF or 0.1 µg/ml C5a and the percentages of cell adhesion were determined (n=3). (**C**) Cells were allowed to migrate for 6 h towards 0.1 µg/ml SCF or 0.1 µg/ml C5a and the percentages of cell chemotaxis were determined (n=3). * P<0.05, ** P<0.01.

functional discrimination between these mast cell subpopulations is attributed to differences in protease expression and differences in expression of the receptor for C5a, C5aR. Tryptase^{high} chymase^{high} skin mast cells and lung mast cell subpopulations express C5aR (37, 38); whereas tryptase^{low} chymase^{low} lung mast cell populations do not (37). The content of tryptase and chymase are highest in skin mast cells, followed by LAD2 cells, and least amounts are found in lung mast cells (39). Most of the effects of C5a in immune cells appear to be mediated through C5aR signal transduction, a G protein-dependent mechanism. Although C5a-C5aR has been shown to stimulate mast cells, most of these studies have been performed using either mast cell lines transfected to express the C5aR (40), immature mast cell lines (41, 42), mucosal mast cells such as BMMC with no endogenous C5aR (27), or C5aR-deficient mice and present inconsistent data. We strived to elaborate on these studies and examined the expression and function of C5a receptor in human LAD2 mast cells. Interestingly, we found that human peripheral blood CD34$^+$ cell-derived human mast cells (HuMC) and human mast cell line LAD2 cells did not express the classical C5aR on their cell surface, but expressed the novel C5a receptor, C5L2. The expression of C5L2 was predominantly intracellular in HuMC, whereas LAD2 cells expressed significant levels of C5L2 on their cell surface. Both the cell types expressed C5aR intracellularly. To the best of our knowledge, no published evidence exists on the C5aR and C5L2 expression in HuMC and LAD2 cells.

C5L2 was identified by Ohno *et al.* as a cDNA with homology to the C5aR (13). Although C5L2 has the conventional structure of a GPCR, studies suggest that C5L2 does not couple conventionally to the known G proteins due to an amino acid replacement of arginine by leucine in the DRY motif located in the third transmembrane domain (16). Due to this structural property C5L2 has been regarded, until recently, as a non-signaling decoy receptor. No mobilization of intracellular Ca^{2+} occurs in C5L2-transfected cells (43, 44), neutrophils from C5aR-deficient mice after stimulation with

C5a (45), or in C5L2-expressing epithelial and myeloid cell lines (46). In the presence of C5a and C5a desArg, C5L2-transfected RBL-2H3 cells do not degranulate (43). Further, C5a binding to C5L2 in bone marrow cells derived from C5aR-deficient mice fails to induce any changes in mRNA expression (44). Recent studies, however, suggest that C5L2 can mediate biological activities of C5a. In a rat model of sepsis induced by cecal ligation and puncture, antibody blockade of C5L2 dramatically increases the level of circulatory IL-6 (47). $C5L2^{-/-}$ mice suffer from augmented inflammatory responses and higher numbers of infiltrating neutrophils in a model of pulmonary immune complex injury (48), indicating an anti-inflammatory function of C5L2. On the contrary, a strong reduction of other inflammatory mediators, such as IL-1β, MIP-1α and MIP-2 is observed in $C5L2^{-/-}$ mice compared to wild types control animals. Furthermore, $C5L2^{-/-}$ mice or mice in which C5aR or C5L2 are blocked by anti-receptor antibody show a higher survival rate in mid-grade sepsis. Additionally, signaling through C5L2 but not C5aR, lead to release of the inflammatory protein, high mobility group box 1 (HMGB1) (49). Inflammatory responses are reduced in the $C5L2^{-/-}$ murine models of peritonitis, thioglycollate-induced migration of neutrophils into dorsal air pouches, and ovalbumin-induced airway hyperresponsiveness (50), supporting a proinflammatory role for C5L2. In human neutrophils, C5L2 functions as an intracellular receptor and negatively modulates C5aR-mediated signaling through the β-arrestin pathway (51). Overall the data points to a more complex role of C5L2 in inflammation in which C5L2 acts not only as a non-signaling decoy receptor but also as positive modulator of C5aR. Most cellular studies of C5L2 also have been performed using transfected cells. Here we utilized LAD2 cells that endogenously expresses the C5L2 receptor and not C5aR and investigated the role of C5L2 in the physiological functions of mast cells. Consistent with the previous reports, we found that C5a did not degranulate LAD2 human mast cells. C5a was also unable to induce Ca^{2+} mobilization. Mast cell-derived mediators are

classified into three categories: granule-associated preformed mediators, *do novo* synthesized lipid mediators, and cytokines and chemokines (52). The release of preformed and *de novo* synthesized mediators occurs within seconds to minutes following mast cell activation, whereas the production of cytokines and chemokines requires transcription and translation of their mRNA and is a comparatively 'late' response (53). It is known that several ligands of GPCRs can differentially activate some or all of the mast cell responses (54). Indeed, incubation of LAD2 cells with C5a for 24 h augmented the production of cytokines and chemokines. C3a and C5a distinctly regulate chemokine production in mast cells by activating divergent signaling pathways (24), possibly by G protein-dependent and -independent mechanisms.

In the present study, C5a induced mast cell chemotaxis, which was compared with chemotaxis towards the known stimuli SCF and C5a was a more potent chemoattractant than SCF for LAD2 cells. Chemotaxis of human mast cells towards C5a was found to be dependent upon binding to fibronectin, which is in agreement with the study of Hartmann *et al*, who showed that C5a-stimulated HMC-1 cells adhere to fibronectin and laminin (22). Fibronectin is an important extracellular matrix that binds to membrane-spanning receptor proteins called integrins and plays a role in cell adhesion, growth, migration, and differentiation (55). Preincubation of mast cells with C5a increased the cell surface expression of CD29 (receptor for fibronectin, also known as integrins) but not ICAM-1, indicating that C5a-mediated mast cell adhesion to fibronectin occurs through the integrins.

To confirm the functional role of C5L2 in human mast cells, we utilized lentivirus shRNA to stably knockdown of expression of C5L2. Using this approach, we uncovered a specific role for C5L2 in modulating human mast cell functions. C5L2 was required for C5a enhancement of cytokine and chemokine production, since the knockdown of the receptor caused the expression levels to return to baseline levels (untreated cells).

Similarly, knockdown cells were unable to adhere to fibronectin or chemotax towards C5a.

The absence of intracellular Ca^{2+} mobilization has suggested that C5L2 is uncoupled from G proteins and must therefore be a non-signaling receptor (13, 43). By contrast, our data showed that C5L2 is functional in mast cells and that it transduces signals in a G protein-dependent manner. Pertussis toxin, which is an inhibitor of the $G\alpha_i$ subunit (56), reduced mast cell adhesion to fibronectin, though the effect was only partial, even at high concentrations of pertussis toxin. The fact that exceptions to the DRY motif paradigm have been reported and that not all the G proteins are known to us, support our observation. Alternatively, changes in Ca^{2+} levels may not be involved in the C5a-activated C5L2 pathway; there are many downstream effectors of G protein activation, many of which can be Ca^{2+}-independent. C3a desArg mediates activation of PLC, PI3K, and ERK1/2 and Akt phosphorylation through C5L2 (57). Moreover, C5a interactions with C5L2 enhance release of G-CSF from lipopolysaccharide-activated mouse peritoneal elicited macrophages via Akt and MEK1/2 (58). Indeed, we showed that C5a-induced mast cell adhesion to fibronectin was sensitive to the inhibitor of PI3K. The PI3K family of lipid kinases play a crucial role in multiple mast cell biological responses, such as degranulation, cytokine release, mast cell homing and homeostasis (59). Various GPCR agonists utilize PI3K to mediate subsequent downstream signaling events (60). In addition to the PI3K pathway, many GPCRs utilize the mitogen-activate protein kinase cascade, including ERK1/2. These signaling pathways are implicated in a variety of cellular responses including chemotaxis, proliferation and differentiation. We demonstrated that C5a induced ERK1/2 phosphorylation in LAD2 cells was sustained for 10 min, further confirming the involvement of G protein signaling in C5L2 function.

Ligand-induced receptor internalization is a central feature of functional seven transmembrane receptors (61). We have clearly demonstrated that C5a stimulated C5L2

internalization by human mast cells. The half-life of C5L2 on the cell surface is considerably longer than that of C5aR and C5aR internalizes and recycles much more rapidly when compared to C5L2 (17, 43). In adipocytes, C5a-induced C5L2 internalization is only detectable after 15 min, and it reaches a maximum at around 1 h in C5L2-HEK cells (18). PMN cells show a decrease in C5L2 protein after 3 h of C5a treatment (62). Consistent with these findings, we showed C5a-mediated C5L2 internalization beginning at 15 min and reaching a maximum at 30 min.

Not only did we demonstrated that C5a activated human mast cells via C5L2, but upregulation of C5L2 with no change in C5aR expression in human mast cells co-cultured with fibroblasts resulted in augmentation of C5a-mediated adhesion and chemotaxis, further confirming that these functions were exerted through activation of C5L2 receptor. Similarly, Th2 cytokine IL-4 increased the expression of C5L2 by human mast cells, highlighting the importance of this receptor in propagation of inflammation.

In summary, our study demonstrated that human mast cells express a functional $G\alpha_i$-coupled stimulatory C5L2 receptor, activation of which promotes mast cell adhesion, chemotaxis, and release of cytokines and chemokines. Thus, C5L2 might be a novel target for modulation/regulation of mast cell responses in allergic inflammation. Given the potential therapeutic value of inhibiting C5L2 function to prevent inflammation as demonstrated in murine models of sepsis, further study of this receptor in the *in vivo* mast cell models is warranted.

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CHAPTER 4. ADENOSINE RECEPTOR A_{2A} INHIBITS COMPLEMENT-MEDIATED ACTIVATION OF HUMAN MAST CELLS BY ACTIVATING $G\alpha_s$ PROTEINS

A version of this chapter has been submitted for publication in the Journal of Biological Chemistry as follows:

P Pundir, S Stredulinsky, A Catalli, M Kulka. Adenosine receptor A_{2A} inhibits complement-mediated activation of human mast cells by activating $G\alpha_s$-proteins. [3]

4.1 ABSTRACT

The complement component 3a (C3a) and adenosine receptors are implicated in the inflammatory process associated with allergic rhinitis and asthma, although a direct interaction between these pathways has not been demonstrated. To investigate the interaction between these pathways the human mast cell line (LAD2) was stimulated with C3a with or without adenosine receptor agonists and antagonists. The non-selective adenosine receptor agonist, 5'-N-(ethylcarboxamido)adenosine (NECA; 10 µg/ml), inhibited C3a-induced LAD2 cell migration, adhesion, degranulation, and production of monocyte chemotactic protein-1. A selective A_{2A} receptor agonist, CGS 21680, inhibited C3a-mediated degranulation but the A_{2B} and A_3 receptor agonists, BAY 60-6583 and IB-MECA, respectively, had no effect. Moreover, an A_{2A} receptor antagonist, SCH 58261, blocked the inhibitory effect of NECA on C3a-induced degranulation, suggesting that inhibition of degranulation was mediated through the A_{2A} receptor. Real-time PCR analysis showed that LAD2 expressed mRNA for A_{2A}, A_{2B} and A_3 receptors but not the A_1 receptor. Measurements of intracellular cyclic adenosine monophosphate (cAMP) showed that NECA elevated [cAMP]i levels in LAD2 cells. Treatment with an activator of

[3] P Pundir designed and performed the experiments, analyzed the data and wrote the chapter; A Catalli and S Stredulinsky assisted with the cAMP and degranulation assays; M Kulka designed the study and experiments, supervised the work and edited the manuscript.

adenylyl cyclase, forskolin, also diminished C3a-induced mast cell degranulation, indicating the involvement of the cAMP signaling pathway in the inhibitory effects of adenosine. Pertussis toxin blocked C3a-activated degranulation. The adenylyl cyclase inhibitor, SQ 22536, had no effect on C3a-activated degranulation but blocked the effect of NECA, demonstrating that C3a and NECA exert their effect through $G\alpha_i$ and $G\alpha_s$ proteins, respectively. Overall, the results suggest that adenosine inhibits complement activation of human mast cells through a $G\alpha_s$-protein pathway.

4.2 INTRODUCTION

Complement component C3a is produced during the complement activation cascade, and belongs to the anaphylatoxin group of proteins along with C4a and C5a (Chapter 1.5.2, Figure 1.8). C3a is a powerful chemoattractant and activator of myeloid cells (1-4), inducing cytokine production, degranulation and respiratory burst in neutrophils, which results in enhanced vascular permeability and local blood flow (5, 6). It is indicated in the pathophysiology of hypersensitivity diseases. Positive correlations have been reported between the extent of complement activation, as assessed by measuring C3a levels or cleavage of C3, and the severity of systemic anaphylaxis in human subjects and animal models of immediate hypersensitivity. An increase in C3a levels have been observed in the serum of asthmatic patients following allergen-induced bronchospasm (7), plasma of patients with aspirin-induced asthma (8), and bronchoalveolar lavage fluid of asthmatic patients after segmental allergen provocation (9). C3a activation is also known to perpetuate the inflammatory reaction in allergic rhinitis (10). C3a mediates its inflammatory activities by interacting with a cell surface cognate G protein-coupled receptor (GPCR), C3aR, on target cells (11-13, Chapter 1.5.2.1).

Mast cells play a central role in the pathogenesis of allergic rhinitis, asthma, and other hypersensitivity/atopic diseases. Through degranulation of preformed mediators and *de novo* production of lipid mediators and cytokines, mast cells are the major cell type responsible for the acute and sometimes life-threatening manifestations of allergic disorders (14, 15, Chapter 1.2.10). Activation by C3a is important to their role in allergic diseases; however, C3aR expression is restricted to only certain types of mast cells. Human mast cell lines HMC-1 (16) and Laboratory of Allergic Diseases 2 (LAD2) (17), primary $CD34^+$ cell-derived mast cells (17, 18) and skin mast cells (19) express C3aR, while murine bone marrow-derived mast cells (BMMC) and rat RBL-2H3 mast cell-like

cells (20) do not and therefore do not degranulate or produce cytokines in response to C3aR activation. Furthermore, mast cell contact with airway smooth muscle cells enhances C3aR-mediated mast cell degranulation (21), suggesting that *in vitro* studies with isolated mast cells might underestimate the *in vivo* importance of these pathways. Independent of C3aR however, C3a peptide derivatives can bind the β subunit of the FcεRI receptor and inhibit its phosphorylation and subsequent signaling events (22), suggesting that the absence of complement receptor expression does not necessarily preclude complement's effects on mast cell functions.

Like complement receptors, adenosine receptors are G-protein coupled and have been indicated in the pathophysiology of allergic inflammation (23, Chapter 1.5.3). Adenosine is an intermediate product of adenine nucleotide metabolism and serves in many organs as a response metabolite in hypoxia or situations where energy consumption is increased. Adenosine, produced in high concentrations during tissue injury, ischemia, or tumor growth, has been implicated in promoting innate immune inflammation (24). Adenosine acts through the four extracellular receptors A_1, A_{2A}, A_{2B}, and A_3 (25, Chapter 1.5.3). Through their ability to inhibit or enhance mediator release from mast cells, these receptors have emerged as novel therapeutic targets in asthma (26). The human mast cell line HMC-1 expresses functional A_{2A} and A_{2B} receptors and produces interleukin (IL)-8 when stimulated with adenosine (27). Studies with primary human mast cells have demonstrated that adenosine can potentiate mast cell degranulation induced by other stimuli such as immunoglobulin (Ig)E and calcium ionophore (28), but that it is not able to act alone. Moreover, adenosine has also been shown to inhibit degranulation (29) in some studies (Chapter 1.5.3.5). The role of adenosine in complement-mediated mast cell activation is unknown. We hypothesized that activation of adenosine receptors would modulate complement-mediated mast cell activation. This study addressed the overall hypothesis of this thesis which is that human

mast cells express both stimulatory and inhibitory GPCR which signal through $G\alpha_i$ or $G\alpha_s$, respectively, and that these receptors are important in chemotaxis and mast cell migration. We chose C3aR as a model for a $G\alpha_i$-coupled stimulatory receptor whose expression, function and signal transduction in human mast cell is well-established. Adenosine receptors were chosen as a model to study stimulatory or inhibitory receptors in GPCR cross-talk.

4.3 METHODS

4.3.1 Cell culture

LAD2 human mast cell line (30) was cultured in serum-free medium (StemPro-34 SFM, Life Technologies, Burlington, ON, Canada) supplemented with 2 mM $_L$-glutamine, 100 U/ml penicillin, 50 µg/ml streptomycin and 0.1 µg/ml stem cell factor (SCF; Peprotech, Rocky Hill, NJ, USA). The cells were maintained at a density of 0.1×10^6 cells/ml at 37℃/5% CO_2, and were periodically tested for expression of Kit and FcεRI by flow cytometry.

4.3.2 Isolation of RNA and generation of cDNA by reverse transcription

Total RNA was isolated using the Tri Reagent method (Sigma-Aldrich Canada, Oakville, ON, Canada). In all, 1 µg of total cellular RNA was reverse-transcribed to cDNA using M-MLV Reverse Transcriptase (Life Technologies) in a 20-µl reaction mix, according to the manufacturer's recommendation.

4.3.3 Real-time quantitative PCR (qPCR)

Gene expression was analyzed using qPCR on a StepOnePlus system (Applied Biosystems, Foster City, CA, USA). For each qPCR assay, a total of 50 ng of cDNA was used. Primer sets (**Table 4.1**) for PCR amplifications were designed using the Primer

Express software. All reactions were performed in triplicate for 40 cycles as per the manufacturer's recommendation. All data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) internal controls (31) and are reported as fold over GAPDH expression.

4.3.4 Flow cytometric analysis

Cells were washed, suspended at 2×10^5 cells/ml in 0.1% bovine serum albumin (BSA)/phosphate-buffered saline (PBS), blocked with 3% BSA/PBS, and stained for 1 h with either a phycoerythrin (PE)-labeled anti-C3aR antibody (R&D Systems, Minneapolis, MN, USA) or an isotype control antibody at 4℃. Cells were washed twice, resuspended in 0.1% BSA/PBS and analyzed on a FACSArray (BD Biosciences, Mississauga, ON, Canada).

4.3.5 Degranulation assay

LAD2 cells were washed, resuspended in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.38 mM $Na_2HPO_4.7H_2O$, 5.6 mM glucose, 1.8 mM $CaCl_2.H_2O$, 1.3 mM $MgSO_4.7H_2O$, 0.4% BSA, pH 7.4) at 2.5×10^4 cells per well and stimulated for 30 min with serial dilutions of C3a (Calbiochem, Billerica, MA, USA) at 37℃/5% CO_2. In some cases, the cells were pretreated with various agonists and antagonists (**Table 4.2**). β-hexosaminidase released into the supernatants and in total cell lysates solubilized with 0.1% Triton X-100 was quantified by hydrolysis of p-nitrophenyl N-acetyl-β-D-glucosamide (Sigma-Aldrich, Oakville, ON, Canada) in 0.1 M sodium citrate buffer (pH 4.5) for 90 min at 37℃/5% CO_2. The percentage of β-hexosaminidase release was calculated as a percent of total content.

Table 4.1 Sequences of oligonucleotides used for real-time qPCR

Gene	Forward primer	Reverse primer	Probe: FAM/TAMRA (GAPDH: MAX/BHQ)
GAPDH	5'-TCG TGG AAG GAC TCA TGA C-3'	5'-CCA TCA CGC CAC AGT TT-3'	5'-/5MAXN/AGT CCA TGC CAT CAC TGC CAC/3IABlk_FQ/-3'
GM-CSF	5'-CAG CCC TGG GAG CAT GTG-3'	5'-ATT CAT CTC AGC AGC AGT GTC TCT A-3'	5'-/56-FAM/AGG CCC GGC GTC TCC TGA ACC/36-TAMSp/-3'
IP-10	5'-CGA TTC TGA TTT GCT GCC TTA TC-3'	5'-TGA TTA CTA ATG CTG ATG CAG GTA CA-3'	5'-/56-FAM/TGG CAT TCA AGG AGT ACC TCT CTC TAG AAC CGT/36-TAMSp/-3'
MCP-1	5'-TCT CTG CCG CCC TTC TGT-3'	5'-GCC TCT GCA CTG AGA TCT TCC-3'	5'-/56-FAM/CTG CTC ATA GCA GCC ACC TTC ATT CCC/36TAMSp/-3'
MIP-1β	5'-CAG CGC TCT CAG CAC CAA-3'	5'-TTC CTC GCG GTG TAA GAA AAG-3'	5'-/56-FAM/CTC AGA CCC TCC CAC CGC CTG C/36-TAMSp/-3'
TGF-β	5'-CTC TCC GAC CTG CAA CAG A-3'	5'-AAC CTA GAT GGG CGC GAT CT-3'	5'-/56-FAM/CCC TAT TCA AGA CCA CCC ACC TTC TGG T/36-TAMSp/-3'
TNF	5'-TCT GGC CCA GGC AGT CA-3'	5'-GCT TGA GGG TTT GCT ACA ACA TC-3'	5'-/56-FAM/ CTT CTC GAA CCC CGA GTG ACA AGC C/36T-AMSp/-3'

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; IP-10, inducible protein-10; MCP-1, monocyte chemoattractant protein-1; MIP, monocyte inflammatory protein; TGF-β, transforming growth factor-beta; TNF, tumor necrosis factor.

Table 4.2 Agonists and antagonists used in this study

Compound	Specificity	Concentration(s) used	Supplier
Pertussis toxin	$G\alpha_i$ protein	0.01, 0.1, 1 µM	Sigma-Aldrich
Wortmanin	PI3K	0.1, 0.5, 1 µM	Sigma-Aldrich
H-89	PKA	0.1, 1, 10 µM	Sigma-Aldrich
Ro-31-8220	PKC	0.1, 0.5, 1 µM	Sigma-Aldrich
NECA	AR	0.01, 0.1, 1, 10 µg/ml	Sigma-Aldrich
CGS 21680	A_{2A} agonist	0.01, 0.1, 1, 10 µM	Tocris Bioscience
BAY 60-6583	A_{2B} agonist	0.01, 0.1, 1, 10 µM	Tocris Bioscience
IB-MECA	A_3 agonist	0.01, 0.1, 1, 10 µM	Sigma-Aldrich
SCH 58261	A_{2A} antagonist	1, 10, 100 µM	Sigma-Aldrich
SQ 22536	Adenylyl cyclase	10 µM	Tocirs Bioscience

AR, adenosine receptors; NECA, 5′-(N-ethylcarboxamido) adenosine, PKA, phosphokinase A; PKC, phosphokinase C; PI3K, phosphoinositol 3-kinase.

4.3.6 Intracellular Ca^{2+} mobilization

LAD2 cells were loaded for 30 min with 1 μM fura-2 acetoxymethyl (AM) (Life Technologies) in HEPES buffer, washed and incubated for 15 min in BSA-free HEPES at 37°C/5% CO_2. A total of 4×10^6 cells were placed in a glass-bottom culture dish under an inverted microscope (Axiovert 200, Carl Zeiss Canada Ltd., Canada). Fura-2 was excited at 340 and 380 nm alternately. The Ca^{2+} response was recorded at 100 ms intervals using SlideBook for Stallion, version 4.26.04 software (Intelligent Imaging Innovations, USA). In all, 0.1 μg/ml C3a was added at the 70 s time point and 1 μM ionomycin (Sigma-Aldrich) at the 256 s time point. The Ca^{2+} response of 20 randomly selected cells was analyzed for each experiment and plotted as 340/380 ratio vs. time.

4.3.7 ELISA

In all, 1×10^6 cells were stimulated for 24 h with 0.1 μg/ml C3a at 37°C/5% CO_2. Cell-free supernatants were isolated and analyzed for cytokine content using the following commercial competitive enzyme linked immunosorbent assay (ELISA) kits: human tumor necrosis factor (TNF)-alpha quantikine ELISA kit, human granulocyte-macrophage colony-stimulating factor (GM-CSF) quantikine ELISA kit, human interleukin-3 (IL-3) quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA). The minimum detection limits were TNF, 5.5 pg/ml; GM-CSF, 3 pg/ml; and IL-3, 7.4 pg/ml.

4.3.8 Cytometric bead array

In all, 0.3×10^6 cells were stimulated for 24 h with 0.0001-0.1 μg/ml C3a at 37°C/5% CO_2. Cell-free supernatants were isolated and analyzed for chemokine content using the human chemokine cytometric bead array (CBA) kit (BD Biosciences). The minimum detection levels were inducible protein-10 (IP-10), 2.9 pg/ml; monocyte chemoattractant protein-1 (MCP-1), 2.7 pg/ml; monokine induced by interferon-γ (MIG),

2.5 pg/ml; RANTES (regulated on activation, normal T cell-expressed and secreted), 1 pg/ml; and IL-8, 0.2 pg/ml.

4.3.9 Adhesion assay

Maxisorp 96-well plates (NUNC, Naperville, IL, USA) were coated for 16 h with 10 µg/ml human fibronectin (Sigma-Aldrich) in PBS at 4°C, washed three times with PBS, blocked for 1 h with 3% BSA in HEPES buffer at 37°C/5% CO_2 and then washed three times with HEPES buffer. Cells were washed with HEPES buffer, suspended at 1 × 10^6 cells/ml, and labeled for 20 min with 5 µM calcein-AM (Life Technologies) at 37°C/5% CO_2. After labeling, cells were washed and resuspended at 1 × 10^6 cells/ml in HEPES buffer. Cell suspension (5 × 10^4 cells) ± HEPES buffer containing 0.1 µg/ml SCF or 0.1 µg/ml C3a was added and incubated for 2 h at 37°C/5% CO_2. In some experiments, cells were pretreated for 30 min with NECA. After incubation, non-adherent cells were washed away with warm HEPES buffer. Fluorescence emission at 530 nm (485 nm excitation) was measured using a fluorescence plate reader (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, Nepean, ON, Canada).

4.3.10 Chemotaxis assay

Performed using the 96-well monocyte cell migration kit (Calbiochem) with slight modifications. Briefly, cells were incubated overnight in SCF-free media, washed with HEPES buffer, and suspended at 0.25 × 10^6 cells/ml. Agonists (0.1 µg/ml SCF or 100 ng/ml C3a) were added to the lower chamber of the 96-well tray and cell suspension (2.5 × 10^4 cells) was added to the insert (upper chamber). Cells were allowed to migrate towards the agonists for 6 h at 37°C/5% CO_2. In some experiments, the cells were pretreated for 30 min with 10 µg/ml NECA. After incubation, the insert was washed twice with warm HEPES buffer. The cells were labeled for 30 min with 5 µM calcein-AM at 37°C/5% CO_2. After labeling, the cells that had migrated across the insert membranes

were lysed using 0.01% Triton X-100. Fluorescence emission was measured at 530 nm (485 nm excitation).

4.3.11 Cyclic AMP (cAMP) assay

In all, 1×10^6 cells were stimulated for 30 min with 0.1-1000 µg/ml NECA or 0.1-10 µg/ml forskolin (Sigma-Aldrich) at 37℃/5% CO_2 and treated with 0.1 µM HCl for 20 min at RT. Cell-free supernatants were analyzed for cAMP accumulation using the cyclic AMP EIA kit (Cayman Chemical, Ann Arbor, Michigan, USA). The minimum detection limit is 3 pmol/ml.

4.3.12 Statistical analysis

Each experiment was performed at least three separate times. Statistical significance was determined by two-tailed Student's paired *t*-test and $P<0.05$ was considered significant. The results are shown as mean ± S.E.M.

4.4 RESULTS

4.4.1 Human mast cells express C3a and adenosine receptors

To confirm the expression of C3aR by human mast cells, we performed flow cytometry using a C3aR-specific antibody. LAD2 cells expressed C3aR on the cell surface (**Figure 4.1A**). As shown in **Figure 4.1B**, the mean florescent intensity (MFI) of anti-C3aR antibody-stained LAD2 cells was significantly higher (MFI 1782.1 ± 220.7) than the cells stained with an isotype control antibody (MFI 153.6 ± 3.1). Upon agonist stimulation, GPCRs undergo phosphorylation, leading to receptor desensitization and internalization (Chapter 1.3.5). We thus tested if C3a engagement would stimulate C3aR on mast cell surface leading to internalize. As shown in **Figure 4.1C**, exposure of LAD2

cells to different concentrations of C3a for 30 min resulted in ~54% internalization of C3aR.

We next characterized the expression of adenosine receptor subtypes by human mast cells. Previous studies with mast cells have shown that the stimulatory or inhibitory effects of adenosine on IgE-induced degranulation are mediated by adenosine receptors (Chapter 1.5.3.4). These receptors have not been characterized in LAD2 cells. qPCR analysis showed that LAD2 cells expressed A_{2A}, A_{2B} and A_3 receptors, but not A_1. The expression level of A_{2A} receptor was the highest among the four subtypes ($A_{2A} > A_{2B} > A_3$) (**Figure 4.1D**).

4.4.2 Adenosine inhibits C3a-induced mast cell degranulation and mobilization of intracellular Ca^{2+}

Adenosine and adenosine receptor subtypes have been shown to differentially limit antigen-induced mast cell activation (Chapter 1.5.3.4). To determine whether adenosine modulated C3a-mediated activation of human mast cells, we utilized a non-selective adenosine receptor agonist, 5'-N-ethylcarboxamidoadenosine (NECA). LAD2 cells were treated with various concentrations of NECA prior to stimulation with C3a and degranulation was assessed by the release of β-hexosaminidase enzyme (Chapter 1.2.6.1). As shown in **Figure 4.2A**, NECA alone did not induce LAD2 cell degranulation. Exposure for 30 min to NECA resulted in inhibition of C3a-induced degranulation and the effect was concentration-dependent. LAD2 cells treated with 0.1-10 µg/ml NECA showed significant reductions in β-hexosaminidase release.

To determine if the inhibition of degranulation by NECA was due to toxicity, we incubated LAD2 cells with 0.1, 1, and 10 µg/ml NECA and measured the metabolic activity of cells using the XTT assay. There was no change in cell metabolic activity following 0.5, 3 or 24 h treatments (data not shown).

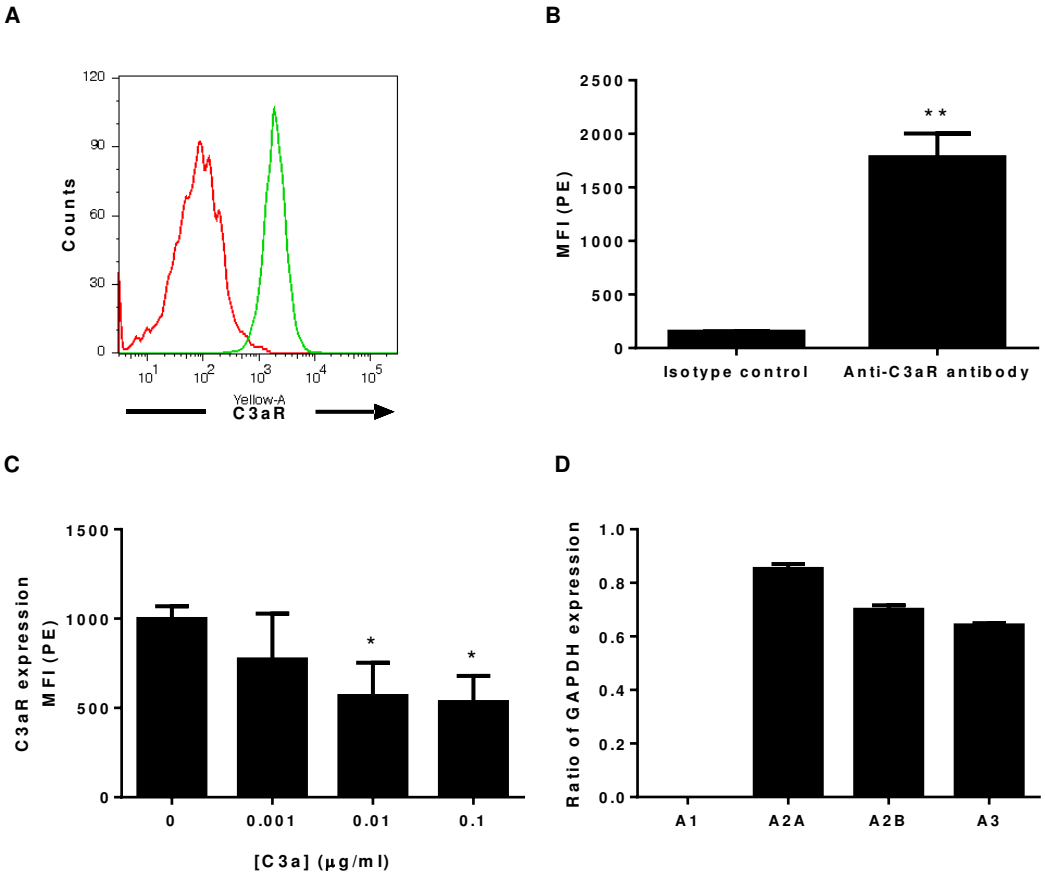

Figure 4.1 C3a and adenosine receptor expression by human mast cells. (**A**) The expression of C3aR on the LAD2 cell surface was measured using flow cytometry with phycoerythrin (PE)-labeled mouse IgG isotype control (red histogram) and PE-anti-C3aR antibodies (green histogram). (**B**) Results of flow cytometric analysis expressed as differences in MFI between LAD2 cells stained with PE-isotype control or PE-anti-C3aR antibody (n=3). (**C**) C3a induces C3aR internalization. LAD2 cells were exposed to indicated concentrations of C3a for 30 min, incubated with PE-isotype control or PE-anti-C3aR antibody and cell surface C3aR expression was analyzed using flow cytometry (n=3). (**D**) Total RNA was isolated from LAD2 cells and A_1, A_{2A}, A_{2B}, and A_3 receptor mRNA expression was examined by qPCR. The data was normalized to GAPDH, and is reported as ratio of GAPDH expression (n=4). * P<0.05, ** P<0.051 compared with untreated.

Mobilization of intracellular Ca^{2+} is essential for mast cell degranulation and incubation of mast cells with C3a has been shown to elicit transient increases of intracellular Ca^{2+} (Chapter 1.5.2.3). As NECA inhibited C3a-induced degranulation, we determined its effect on intracellular Ca^{2+}. LAD2 cells loaded with the Ca^{2+}-sensing fluorescent dye fura-2 AM showed a rapid increase in intracellular Ca^{2+}, reaching a peak within 70 s after C3a exposure. As expected, ionomycin used as a positive control caused additional Ca^{2+} influx. Pretreatment with 10 µg/ml NECA for 30 min significantly blocked C3a-induced mobilization of intracellular Ca^{2+} in LAD2 cells (**Figure 4.2B-C**). No inhibitory effect was seen on ionomycin-induced Ca^{2+} flux.

4.4.3 Adenosine inhibits C3a-induced production of cytokines

Mast cell-derived cytokines and chemokines propagate inflammatory reactions (Chapter 1.2.6.3). C3a is known to induce their production by mast cells (Chapter 1.5.2.3). We therefore determined if adenosine would modulate the effect of C3a on cytokine release from LAD2 cells. While C3a caused no change in mRNA expression of the indicated cytokines (**Figure 4.3A**), mRNA expression levels of the chemokines MCP-1/CCL2 and IP-10/CXCL10 increased significantly (**Figure 4.3B**). To confirm our observation that C3a preferentially activated chemokine production in LAD2 cells, we performed ELISA for TNF, GM-CSF, and IL-3 (**Figure 4.3C**), and CBA for IP-10, MCP-1, MIG, RANTES, and IL-8 (**Figure 4.3D**). C3a induced production of MCP-1 (43.3 pg/ml per 300,000 cells) and IP-10 (28.8 pg/ml per 300,000 cells) (**Figure 4.3D**). We next assessed the effect of NECA on C3a-induced MCP-1 production. LAD2 cells were pretreated with NECA for 30 min and activated with C3a for 24 h, and MCP-1 production was then analyzed by ELISA. NECA inhibited C3a-induced MCP-1 production compared with untreated cells (**Figure 4.3E**).

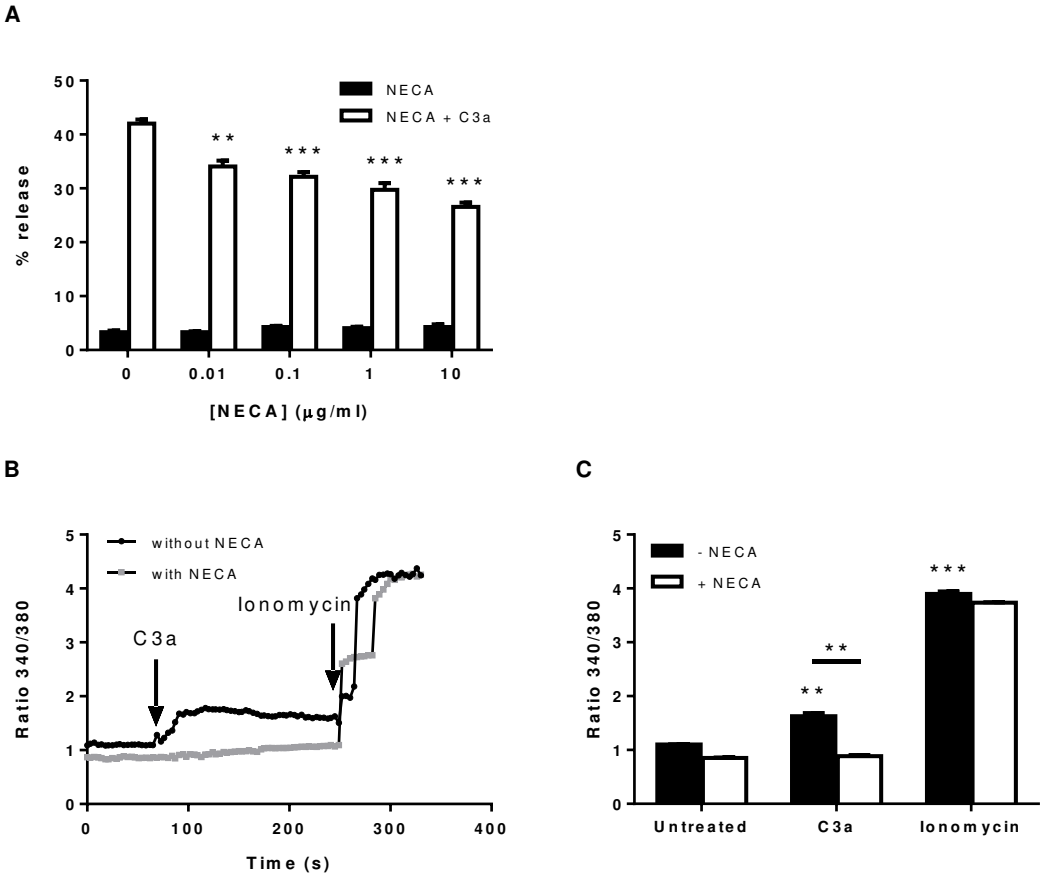

Figure 4.2 The inhibitory effect of adenosine on C3a-mediated degranulation and intracellular Ca^{2+} mobilization. (**A**) LAD2 cells were treated with the indicated concentrations of NECA prior to stimulation with 0.1 µg/ml C3a after which the percentages of β-hexosaminidase release were measured (n=3). (**B**) LAD2 cells were pretreated with for 30 min with 10 µg/ml NECA, loaded with fura-2, stimulated with 0.1 µg/ml C3a at time point 70 s followed with 1 µM ionomycin at time point 256 s and mobilization of intracellular Ca^{2+} was measured (n=3). (**C**) Ca^{2+} response in 20 randomly selected individual LAD2 cells reported as mean ± S.E.M. ** P<0.01, *** P<0.001 compared with untreated.

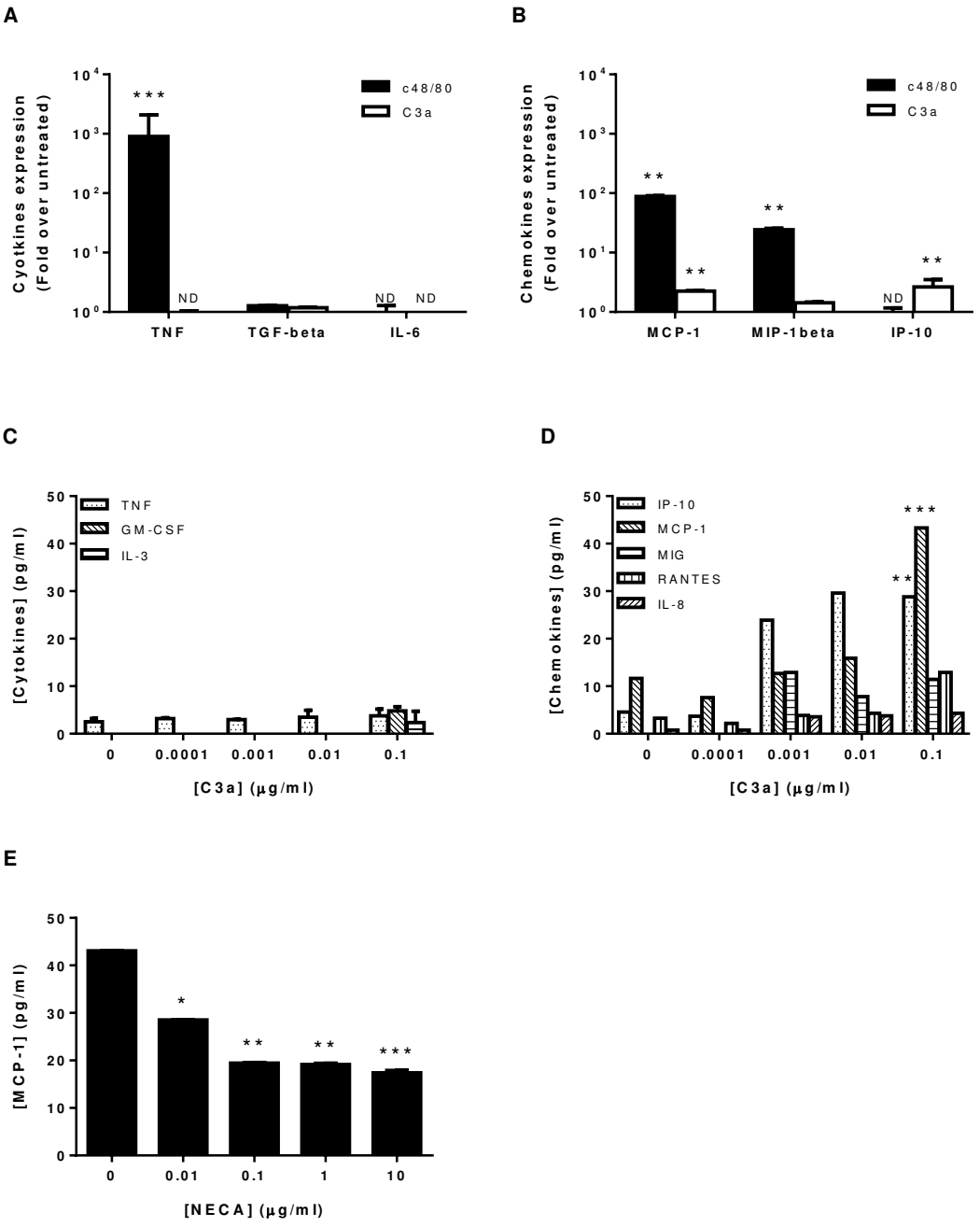

Figure 4.3 Inhibitory effect of adenosine on the production of inflammatory mediators. After 3 h treatment with 0.1 µg/ml C3a, mRNA expression of (**A**) TNF, TGF-β, IL-6, (**B**) MCP-1, MIP-1β, and IP-10 was measured by qPCR (n=3). Data were normalized GAPDH mRNA levels and are expressed as fold increase over unstimulated controls. After 24 h of treatment the levels of (**C**) TNF, GM-CSF, IL-3, (**D**) IP-10, MCP-1, MIG, RANTES, and IL-8 in cell-free supernatants was measured by ELISA or CBA (n=3). (**E**) LAD2 cells were treated with 10 µg/ml NECA prior to stimulation with 0.1 µg/ml C3a, and the levels of MCP-1 in cell-free supernatants were measured by ELISA (n=3). * P<0.05, ** P<0.01, *** P<0.001 compared with untreated.

4.4.4 Adenosine inhibits C3a induced mast cell adhesion and chemotaxis

Activation of mast cell degranulation in allergic inflammation requires their migration and accumulation at sites of infection. C3a can evoke mast cell adhesion, chemotaxis, and subsequent degranulation (Chapter 1.5.2.3). As NECA inhibited mast cell degranulation, we evaluated whether it could also block mast cell migration and adhesion. Comparable with SCF, C3a activated LAD2 cells to adhere to fibronectin, an extracellular matrix protein. Treatment with 10 µg/ml NECA blocked C3a-induced mast cell adhesion to fibronectin (**Figure 4.4A**). Next, we measured the chemotaxis of NECA-treated LAD2 cells towards C3a. Results showed that, similar to SCF, C3a activated LAD2 chemotaxis. Pretreatment with 10 µg/ml NECA blocked C3a-activated cell chemotaxis (**Figure 4.4B**).

4.4.5 Adenosine inhibits human mast cell degranulation through activation of the A_{2A} receptor

After demonstrating that adenosine blocked the effect of C3a on human mast cell chemotaxis, adhesion, degranulation and MCP-1 release, we questioned which adenosine receptor subtype(s) mediated the inhibitory effects of adenosine. As our first set of experiments had revealed that LAD2 cells express A_{2A}, A_{2B} and A_3 receptors, we utilized agonists and antagonists (**Table 4.2**) for these receptors and performed degranulation assays. LAD2 cells were pretreated with the A_{2A} receptor selective agonist CGS 21680, the A_{2B} receptor selective agonist BAY 60-6583, or the A_3 receptor selective agonist IB-MECA prior to activation with C3a and the release of β-hexosaminidase was measured. Pretreatment with CGS 21680 inhibited C3a-induced mast cell degranulation (**Figure 4.5A**). BAY 60-6583 and IB-MECA failed to inhibit C3a-induced degranulation (**Figure 4.5B-C**). We next blocked A_{2A} receptor by SCH 58261, a highly selective A_{2A} receptor antagonist, and then tested the ability of NECA to inhibit C3a-induced

degranulation. As shown in **Figure 4.5D**, SCH 58261 reduced the inhibitory effect of NECA on C3a-induced mast cell degranulation.

To determine if the effects mediated by CGS 21680 and SCH 5826 were due to toxicity, we incubated LAD2 cells with 0.1, 1, and 10 µg/ml NECA and measured the metabolic activity of cells using the XTT assay. There was no change in cell metabolic activity following 0.5, 3 and 24 h treatments (data not shown). Collectively, these results indicate that the inhibitory effect of adenosine on C3a-mediated degranulation of human mast cells is mediated via A_{2A} adenosine receptor.

4.4.6 Inhibitory effect of adenosine is cAMP-dependent

To understand the mechanism by which adenosine inhibits C3a-stimulated mast cell function, we investigated the cAMP levels in human mast cells. LAD2 cells were pretreated with NECA and an ELISA was performed for cAMP. As shown in **Figure 4.6A**, NECA elevated the cAMP levels in LAD2 cells in a concentration-dependent manner. We next utilized forskolin to confirm the role of cAMP signaling in inhibiting C3a effect on human mast cells. Forskolin enhances intracellular cAMP accumulation in cells by directly activating adenylyl cyclase. LAD2 cells were treated with forskolin prior to stimulation with C3a and the degranulation was measured. Forskolin inhibited C3a-induced mast cell degranulation (**Figure 4.6B**). Collectively, these findings suggest that increase in intracellular cAMP is required for inhibition of C3a-mediated mast cell activation by adenosine.

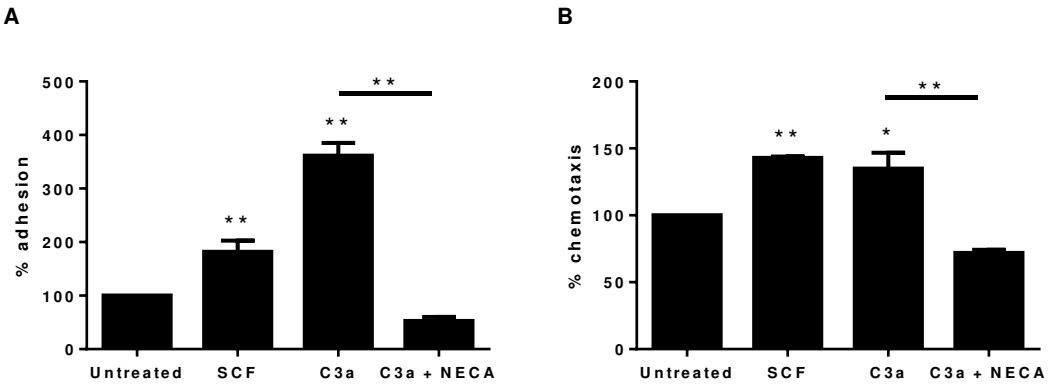

Figure 4.4 Inhibitory effect of adenosine on mast cell adhesion and chemotaxis. (**A**) LAD2 cells were treated with 10 µg/ml NECA for 30 min, allowed to adhere to fibronectin-coated wells for 2 h in the presence of 0.1 µg/ml C3a and the percentages of cell adhesion were assessed. 0.1 µg/ml SCF was used as a positive control (n=3). (**B**) LAD2 cells were treated with 10 µg/ml NECA for 30 min, allowed to migrate towards 0.1 µg/ml C3a for 6 h and the percentage of cell chemotaxis were assessed. 0.1 µg/ml SCF was used as a positive control (n=3). * $P<0.05$, ** $P<0.01$ compared with untreated.

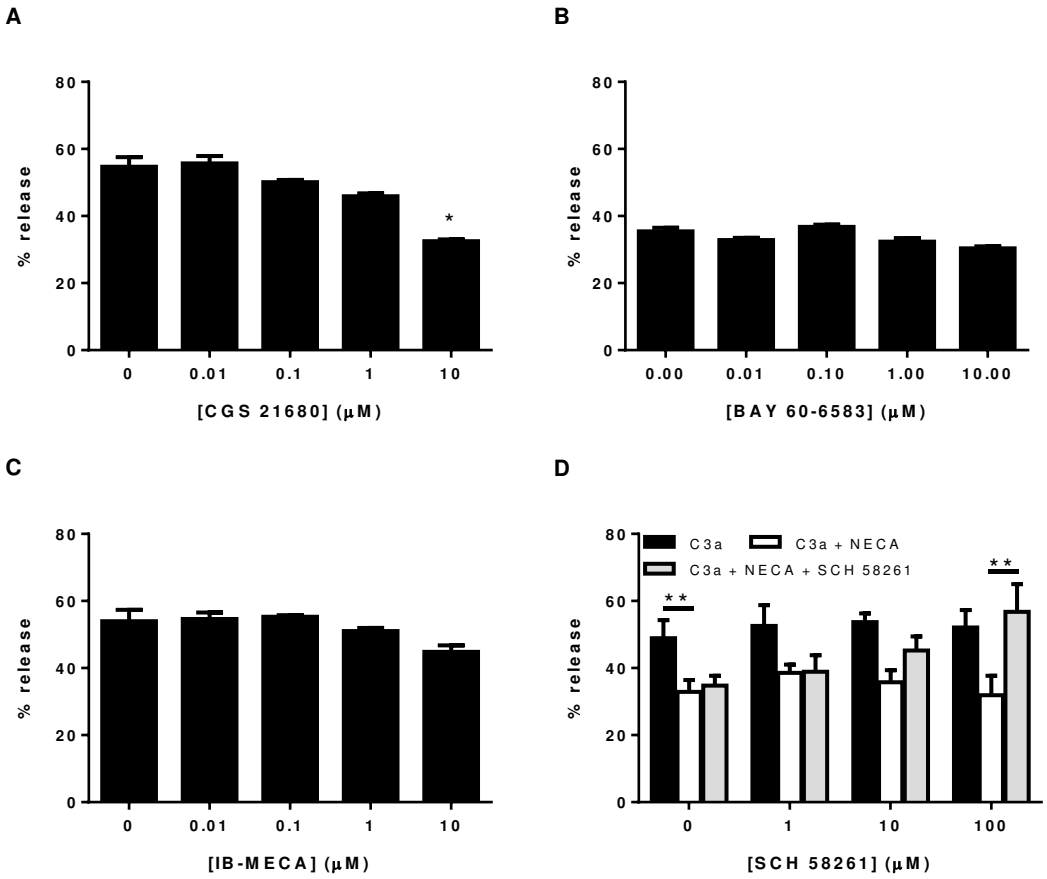

Figure 4.5 Effect of adenosine receptor agonists and antagonist on mast cell degranulation. LAD2 cells were treated for 30 min with the indicated concentrations of (**A**) CGS 21680, (**B**) BAY 60-6583, or (**C**) IB-MECA prior to stimulation with 0.1 μg/ml C3a and the percentages of β-hexosaminidase release were measured (n=3). (**D**) LAD2 cells were treated for 30 min with the indicated concentrations of SCH 58261 followed by 30 min treatment with 10 μg/ml NECA prior to stimulation with 0.1 μg/ml C3a and the percentage of β-hexosaminidase release were measured (n=3). * $P<0.05$, ** $P<0.01$ compared with untreated.

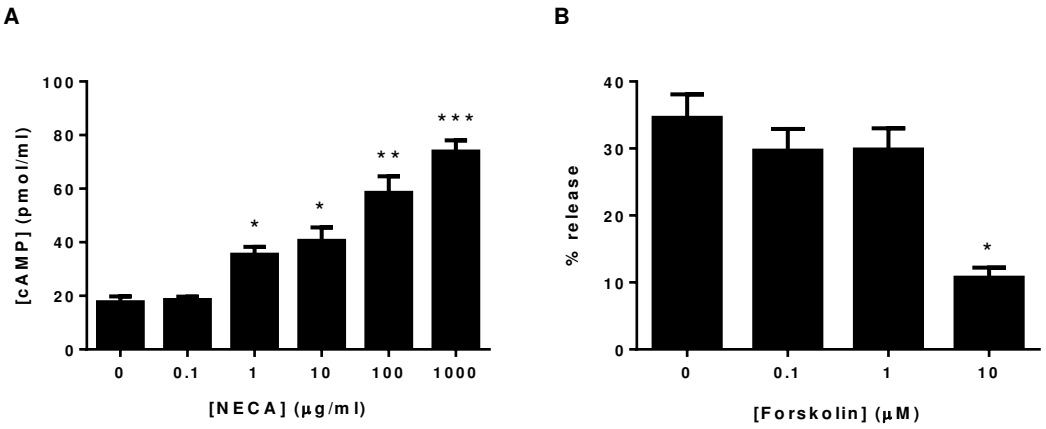

Figure 4.6 The role of cAMP in the inhibitory effects of adenosine on C3a-induced mast cell activation. (**A**) LAD2 cells were treated for 30 min with the indicated concentrations of NECA and the accumulation of cAMP in cell-free supernatants was measured using ELISA (n=5). (B) LAD2 cells were treated for 30 min with the indicated concentrations of forskolin prior to stimulation with 0.1 µg/ml C3a and the percentages of β-hexosaminidase release were measured (n=3). * P<0.05, ** P<0.01, *** P<0.001 compared with untreated.

4.4.7 Adenosine mediates its effect on human mast cells via $G\alpha_s$ protein

$G\alpha_s$-coupled receptors mediate their effects through activation of adenylyl cyclase and subsequent accumulation of cAMP in cells. A_{2A} and A_{2B} receptors stimulate adenylyl cyclase via coupling to $G\alpha_s$ protein, whereas A_1 and A_3 receptors inhibit adenylyl cyclase via coupling to $G\alpha_i$ protein. In our study, LAD2 cells showed highest expression of A_{2A} receptor, activation of which led to inhibition of C3a-induced mast cell degranulation. Moreover, treatment with adenosine elevated cAMP levels in LAD2 cells. Therefore, the findings indicated a functional role of A_{2A} receptor in C3a-activated LAD2 cells. To verify that adenosine modulated human mast cells by $G\alpha_s$-mediated signal transduction, LAD2 cells were pretreated with the adenylyl cyclase inhibitor, SQ 22536 prior to stimulation with NECA or C3a and degranulation was measured. As shown in **Figure 4.7A**, SQ 22536 had not effect on mast cell degranulation induced by C3a. However, it significantly attenuated the inhibitory effect of NECA on C3a-induced degranulation. We also confirmed signal transduction mediated by C3a in human mast cells. LAD2 cells were treated with pertussis toxin, an inhibitor of $G\alpha_i$ prior to stimulation with C3a and degranulation was assessed. Pertussis toxin significantly attenuated degranulation by C3a (**Figure 4.7B**). Collectively, the data demonstrated that adenosine and C3a act via $G\alpha_s$ and $G\alpha_i$ proteins, respectively.

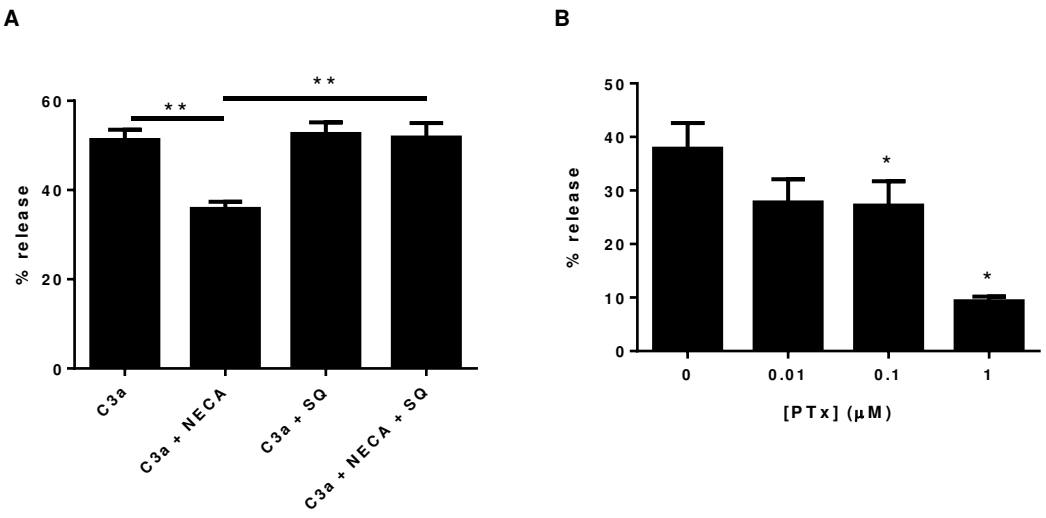

Figure 4.7 The effects of adenosine and C3a on mast cell activation are $G\alpha_s$- and $G\alpha_i$-dependent. (**A**) LAD2 cells were treated for 30 min with 10 µM SCH 58261 followed by treatment for 30 min with 10 µg/ml NECA prior to stimulation with 0.1 µg/ml C3a and the percentages of β-hexosaminidase release were measured (n=3). (B) LAD2 cells were treated for 2 h with the indicated concentrations of pertussis toxin (PTx) prior to stimulation with 0.1 µg/ml C3a and the percentages of β-hexosaminidase release were measured (n=3). * P<0.05, ** P<0.01 compared with untreated.

4.5 DISCUSSION

Complement component C3a is a potent mediator of inflammation and the functions of C3a on various inflammatory cells are mediated through binding to the specific receptor C3aR (Chapter 1.5.2.1). Consistent with the previous reports (17), we showed that the human mast cell line LAD2 expressed C3aR on its cell surface. C3a stimulation degranulated LAD2 cells and stimulated secretion of the proinflammatory mediators MCP-1 and IP-10. In addition, we demonstrated that it stimulated cell adhesion to fibronectin, an important extracellular matrix protein that has been implicated in the migration of immune cells in inflammation. Indeed, we found that C3a facilitated the chemotaxis of human mast cells, an effect which was mediated in a G-protein dependent manner. Contribution of mast cells and their biologically active mediators to allergic inflammation are well described. Mast cell activation by FcεRI is a critical event in allergic inflammation (32, Chapter 1.2.5.1), and growing evidence has demonstrated that mast cell activation via cell-surface GPCRs also plays a crucial role in inflammation (33, Chapter 1.2.5.2). GPCRs can mediate mast cell degranulation, either directly or via the modulation of FcεRI-mediated degranulation (34). Activation of C3a and sphingosine-1-phosphate can induce degranulation directly; however, adenosine and prostaglandin E_2 alone cannot induce degranulation, but they can enhance antigen-induced mast cell degranulation via their cognate GPCR (34). Furthermore, cross-talk between different GPCRs has also been acknowledged to modulate mast cells responses (34, 35, Chapter 1.4). In this study, we elucidated a novel role for adenosine receptor as a negative regulator of C3a-mediated human mast cell activation. LAD2 cells incubated with a potent non-selective adenosine receptor agonist, NECA, displayed reduced β-hexosaminidase release to C3a challenge. The inhibitory effect of NECA on degranulation correlated with decreased calcium influx in mast cells following activation with C3a. Mast cell adhesion to fibronectin, chemotaxis, and MCP-1 and IP-10

production were also reduced in NECA-treated mast cells, suggesting that adenosine receptor signaling may be of importance in regulation of human mast cell activation in inflammation.

Our observations of the inhibitory effect of adenosine on C3a-stimulated mast cell degranulation *in vitro* are consistent with several previous reports on human lung fragments, dispersed human lung mast cells, human umbilical cord blood-derived mast cells and murine BMMC (29, 36-38). These studies have shown that adenosine can inhibit antigen-induced histamine release from mast cells. Our study is the first to characterize the inhibitory effect of adenosine on complement-mediated degranulation. Some studies have shown a biphasic effect of adenosine on antigen-induced human mast cell activation (37, 39). Low concentrations of adenosine potentiate, and high concentrations inhibit antigen-induced degranulation of human mast cells (37). Furthermore, a few studies have reported that adenosine alone can induce release of cytokines from the human mast cell line HMC-1 (27, 40-43). The contrasting effect of adenosine on mast cells and in allergic asthma have been linked to the differential activation of the adenosine receptors.

Adenosine receptors are cell surface GPCR; four subtypes (A_1, A_{2A}, A_{2B}, and A_3) have been described (25, Chapter 1.5.3). A_1 and A_3 subtypes predominantly couple to $G\alpha_{i/o}$ and the A_{2A} and A_{2B} couple to $G\alpha_s$ (25, Chapter 1.5.3.1). We characterized the expression of adenosine receptors by human mast cells and found that LAD2 human mast cell line expressed A_{2A}, A_{2B}, A_3 but not A_1 adenosine receptors. A_{2A} receptor had the highest expression levels compared to A_{2B} and A_3 receptors. This pattern of adenosine receptor expression was different to that shown for BMMC, RBL-2H3, cutaneous mast cells and murine primary lung mast cells, where the A_3 receptor is predominantly expressed. The abundance of A_3 receptor transcripts in these cells might explain the direct effect of adenosine on mast cell degranulation (44-46). We observed

no degranulation of mast cells with NECA per se in the absence of C3a. Moreover, the selective A_{2A} receptor agonist, CGS 21680 and the A_3 receptor agonist, IB-MECA, alone had no direct activity on mast cell degranulation.

Previous studies have shown that the A_1, A_{2B} or A_3 receptors elicit proinflammatory effects in asthma through the activation of histamine and bronchoconstrictive substances release from mast cells, mucus secretion and bronchoconstriction (26, Chapter 1.5.3.5). A_{2A} receptors are believed to elicit anti-inflammatory signals by inhibiting histamine and tryptase release from mast cells *in vitro* (29, 37, 38, 47). *In vivo* administration of an A_{2A} receptor agonist attenuates airway inflammation in ovalbumin-sensitized and –challenged rats (48). Similarly, genetic ablation of the A_{2A} receptor augments airway inflammation and hyperresponsiveness in a murine model of allergic airway inflammation (49), further supporting the role of the A_{2A} receptor as mediating anti-inflammatory functions. We therefore attempted to determine which specific adenosine receptor(s) were involved in inhibition of C3a-mediated activation of human mast cells. As NECA inhibited β-hexosaminidase release from C3a-stimulated mast cells, these findings suggested that A_{2A} or A_{2B} receptor activation evoke inhibitory effects on degranulation of these cells. Indeed, the selective A_{2A} receptor agonist, CGS 21680, inhibited C3a-induced degranulation of human mast cells but the A_3 receptor agonist, IB-MECA, had no effect. NECA, however, failed to inhibit β-hexosaminidase release from mast cells pretreated with A_{2A} receptor antagonist, SCH 58261, but A_{2B} receptor and A_3 receptor antagonists had no effect, further suggesting that the inhibition of degranulation was mediated by A_{2A} receptor.

Pharmacological agents with cyclic adenosine monophosphate (cAMP)-elevating properties have long been observed to inhibit mast cell function (50). cAMP is a ubiquitous intracellular second messenger that is produced from AMP after the activation of adenylyl cyclase. cAMP can inhibit store-operated calcium channels in mast cells

through a PKA-dependent pathway, which subsequently limits antigen-induced mast cell degranulation (51, Chapter 1.5.3.3). Different strategies can raise intracellular cAMP levels, including activation of $G\alpha_s$-coupled receptors (52, 53). Indeed, activation of $G\alpha_s$-coupled A_{2A} and A_{2B} receptors have been shown to increase cAMP levels in mast cells (36, 40). Thus, we addressed the question as to whether the inhibition of degranulation by NECA was cAMP-dependent. We found the treatment with NECA elevated intracellular cAMP levels in human mast cells in a concentration-dependent manner, suggesting that A_{2A}-$G\alpha_s$ signaling may be of critical importance in the regulation of mast cells activation in inflammation. Forskolin also exerts its biological activities by direct stimulation of adenylyl cyclase, thereby increasing cellular concentrations of cAMP (54). Forskolin potentiation of cAMP in turn inhibits basophils and mast cells degranulation and histamine release (55). We therefore utilized forskolin to further confirm the role of constitutive cAMP in regulation of mast cell functions. Similar to NECA, forskolin showed inhibited C3a-mediated mast cell activation. In addition, forskolin also inhibited human mast cell adherence to fibronectin as well as chemotaxis.

To further understand the molecular mechanisms underlying the activities of C3a and NECA, we investigated the requirements for G protein subtypes, as their roles in complement- (56-63) and adenosine-mediated (40) mast cell activation have been previously reported. Pertussis toxin blocked C3a-induced degranulation of human mast cells. The adenylyl cyclase inhibitor, SQ 22536, had no effect on C3a-activated degranulation but blocked the effect of NECA, demonstrating that C3a and NECA mediate their effects through $G\alpha_i$ and $G\alpha_s$ proteins, respectively. Overall, this data confirmed that C3a activates G protein-dependent signal transduction in human mast cells and adenosine inhibits complement activation of human mast cells through a $G\alpha_s$ protein pathway

In summary, this study demonstrated that activation of the $G\alpha_s$-coupled inhibitory A_{2A} adenosine receptor diminishes the activating effect of $G\alpha_i$-coupled C3aR on human mast cells. This provides a critical balance between mast cell activation and mediator release in host defense and inflammation. In cases of unregulated mast cell activation via a GPCR, for instance the C3aR, targeting inhibitory A_{2A} adenosine receptors might prove beneficial. Thus adenosine receptors offer novel therapeutic targets in allergic inflammation and hypersensitivity.

4.6 REFERENCES

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CHAPTER 5. GENERAL DISCUSSION

5.1 SUMMARY

We live in an environment filled with microorganisms that are constantly trying to breach our immune system to gain dominance. The innate immune system acts as a sensor for these pathogens and eliminates them by utilizing a range of defense processes, such as the immune cells. To develop an effective immune response, the cells of the immune system are required to communicate between each other through secretion of soluble mediators and direct cell-cell interactions. Among the cells of the innate immune system, mast cells appear to be among the most powerful in terms of their ability to respond to multiple stimuli and to selectively release different types and amounts of mediators (1). The most studied and well-described (classical) mechanism of mast cell activation is via stimulation of the high-affinity immunoglobulin E (IgE)-receptor, FcεRI. Binding of an antigen to FcεRI-bound specific IgE leads to FcεRI aggregation, which in turn induces downstream signaling events and the release of mediators (2). Previous studies from our laboratory and the data presented in this thesis demonstrate that IgE-antigen stimulation of human mast cells (Laboratory of Allergic Diseases 2 human mast cell line) induced degranulation and release of β-hexosaminidase, cysteinyl leukotrienes (CysLTs) and prostaglandin D_2 (PGD_2). No significant effect was observed on cytokine and chemokine production (3). Whereas the signaling pathways leading to degranulation and production of mediators are relatively well understood, while those involved in IgE-antigen-mediated mast cell chemotaxis are still vague, however the phosphoinositol 3-kinase (PI3K)-dependent pathway appears to contribute to chemotactic response (4).

With the expansion of research on mast cell pathophysiology, our perception of mast cells in immune functions has changed. Mast cells are no longer viewed solely as

mediators of allergic inflammation; their presence serves essential functions in host defense. In fact, their functions extend throughout all of the stages of the immune response, ranging from shaping the response against pathogens, regulating both innate and adaptive immune cell functions, to supporting regulatory cells in the maintenance of tissue-tolerance (1, 5). Mast cells are strategically positioned at the interface with the environment where they can be activated directly by interacting with pathogens through pattern-recognition receptors (PRRs), including Toll-like receptors (TLR), Nod-like receptors (NLRs), C-type lectin receptors such as Dectin-1, and the glycosylphosphatidylinositol (GPI)-anchored protein CD48. PRRs have the ability to recognize pathogens using evolutionarily conserved patterns called pathogen-associated molecular patterns (PAMPs), resulting in opsonization, activation of complement and coagulation cascades, phagocytosis, activation of proinflammatory signaling pathways, and induction of apoptosis (5). Engagement of PRRs leads to selective release of mast cell mediators. For example, peptidoglycan stimulation of mast cells via TLR2 leads to both degranulation and cytokine production, whereas stimulation through TLR3 and TLR4 results in cytokine release alone (6, 7). Furthermore, Dectin-1 binding of fungal β-glucan induces the release of leukotriene LTC_4 by mast cells (8), while CD48 binds to the *E. coli* adhesin FimH and induces the release of tumor necrosis factor (9). Activation of PRR also induces the production of antimicrobial peptides, such as defensins and cathelicidins, which may further regulate host defense against pathogens by serving as an alarm for sentinels, such as mast cells (10).

Antimicrobial peptides are a unique and diverse group of gene-encoded proteins, which are highly conserved in their structure, function and mechanism of action (11). In addition to their antibacterial role, many of the antimicrobial peptides have been shown to interact with immune cells, resulting in activation of innate and adaptive immune responses (11). This conclusion is based on the chemotactic effect of antimicrobial

peptides for selected immune cells. The chemotactic ability of the antimicrobial peptides indicates that they can attract cells, including mast cells, expressing the appropriate receptors along a gradient to their site of origin, Furthermore, as their chemotactic responses can be inhibited by pertussis toxin, the antimicrobial peptides interact with $G\alpha_i$-coupled receptors. The major antimicrobial peptides found in humans are defensins and cathelicidins. The chemotactic effect of cathelicidin for neutrophils, monocytes, and T cells occurs through interaction with N-formyl peptide receptor-like 1 (FPRL1) receptor. Although the chemotactic effect of cathelicidin for mast cells is also pertussis toxin-sensitive, prior to our study the interaction of antimicrobial peptides with FPRL1 in human mast cells was questionable (12). Pleurocidin is a novel α-helical cationic antimicrobial peptide that displays a broad spectrum antibacterial activity along with diverse proinflammatory and immunomodulatory functions. As pleurocidins are structurally and functionally similar to cathelicidins, we chose them for our agonist model for investigating the biological roles of FPRL1 in human mast cells. Our studies showed that some pleurocidins caused human mast cells to adhere, migrate, degranulate and release cysteinyl leukotrienes, prostaglandins, and chemokines. Pleurocidin NRC-04 activated human mast cells by binding and signaling via the FPRLI receptor. FPRL1 is a classical chemoattractant GPCR originally identified in phagocytic leukocytes; it mediates cell chemotaxis and activation in response to bacterial formylated chemotactic peptides (e.g. fMLF). Agonist binding to FPRL1 activates a $G\alpha_i$ protein signal transduction cascade involving PI3K, phospholipase C (PLC), protein kinase C (PKC), mitogen-activated protein kinases (MAPKs) and the transcription factor nuclear factor (NF)-κB (12). Indeed, we demonstrated that pertussis toxin, wortmannin (PI3K inhibitor), U-73122 (PLC inhibitor) and Ro-31-8220 (PKC inhibitor) each blocked pleurocidin-induced mast cell mediator release. Our data described a novel proinflammatory and

stimulatory role for GPCR FPRL1 in mast cell-driven immune response to cationic antimicrobial peptides.

Similar to the antimicrobial peptides, the complement system represents an evolutionarily ancient and conserved part of the innate immune system which is involved in a rapid and nonspecific host response. Activation of the complement system leads to release of multiple proteolytic fragments, including C3a and C5a (13, 14). C3a and C5a are collectively called anaphylatoxins because they induce the release of mediators from mast cells, basophils, which causes a rapid increase in vascular permeability that is a characteristic of anaphylaxis. In addition to their traditional roles in inflammation, anaphylatoxins also significantly influence the innate and adaptive immune responses by activation and modulation of different dendritic cell populations, B cells, and T cells. C5a promotes phagocytosis and the oxidative burst in neutrophils and monocytes, upregulates adhesion molecules like CD11b in neutrophils, and induces the release of granular enzymes. At subnanomolar to nanomolar concentrations, C5a elicits chemotaxis of cells of the myeloid lineage, including neutrophils, monocytes, eosinophils, and basophils. Higher nanomolar concentrations of C5a also elicit degranulation and an oxidative burst (15). The effects of C5a are mediated through two receptors, the C5a receptor (C5aR) and C5a receptor-like 2 (C5L2), which are distributed on immune cells of myeloid origin as well endothelial, epithelial and parenchymal cells. The significance and biological role of C5L2 is debated and not fully understood. We investigated the roles of C5aR and C5L2 in human mast cells for C5a-induced activation. C5a caused human mast cells to adhere, migrate and produce cytokines and chemokines, but not to release β-hexosaminidase, CysLTs and PGD_2. C5a activated human mast cells by signaling via the C5L2 receptor, but not via the C5aR. Furthermore, treatment with pertussis toxin and wortmannin (a PI3K inhibitor)

blocked C5a-induced mast cell adhesion to fibronectin. Our studies thus elucidated a novel stimulatory role for $G\alpha_i$-coupled C5L2 in human mast cell, indicating that the C5L2 receptor may participate in innate immune modulation of inflammation.

Like C5a, C3a participates in homeostasis and inflammation by modulating activities, such as increases in vascular permeability, smooth muscle contraction, immune cell recruitment, and other responses (e.g., chemotaxis and phagocytosis). C3a and its inactivated derivative C3a desArg also possess potent antibacterial activity (16). The functional responses of C3a are mediated by its interaction with C3a receptor (C3aR). Our studies confirmed the previous reports and demonstrated that C3a activated mast cell degranulation and selective release of chemokines only. In addition, we showed that C3a also induced human mast cell chemotaxis and adhesion to fibronectin. Binding of C3a to C3aR resulted in coupling to $G\alpha_i$ proteins, as demonstrated by the inhibitory effect of pertussis toxin on C3a-induced activation of human mast cells. Moreover, treatment with wortmannin (a PI3K inhibitor), H-89 (a PKA inhibitor), and Ro-31-8220 (a PKC inhibitor) blocked the effect of C3a, confirming the activation of stimulatory C3aR-$G\alpha_i$ signal transduction in human mast cells. Simultaneous activation of $G\alpha_s$-coupled adenosine receptor, A_{2A}, inhibited C3a-mediated activation of human mast cell chemotaxis, adhesion, and mediator release. Interestingly, adenosine by itself displayed no effect on mast cell activation. This confirmed our central hypothesis that human mast cells express inhibitory GPCRs which display cross-talk with other GPCR, an important phenomenon in mast cell modulation of innate inflammation.

The data presented in this thesis also confirmed that while the antimicrobial peptides and the complement proteins differentially activated the release of proinflammatory mediators from human mast cells, chemotaxis and adhesion were the

most common responses induced. More importantly, the effects on chemotaxis and adhesion were conserved through the G protein-dependent signaling mechanism. Also, PI3K appeared to be the common molecule utilized downstream of the G protein signaling cascade by the three agonist-GPCR interactions. PI3K is a family of lipid kinases that play crucial roles in multiple biological processes, such as mast cell degranulation and cytokine production. It is also critical for mast cell chemotaxis, adhesion and homeostasis. This has been evidenced by the ability of wortmannin and LY294002 to effectively inhibit antigen-, stem cell factor-, and PAMP-mediated mast cell migration and adhesion to fibronectin-coated plates (4). Consistent with this, in our studies wortmannin blocked adhesion of human mast cells to fibronectin in response to pleurocidin, C5a, and C3a. Due to their central roles in the generation and activation of mast cells, PI3K-regulated pathways are attractive targets for the regulation of mast cells responses in health and disease. PI3K has also been proposed to regulate amplification pathways for ongoing signaling events initiated by the GPCR pathways (4).

As mentioned before, precisely how GPCRs influence mast cell function depends directly upon the class of G protein engaged and the individual subunits (Gα and βγ) that regulate effector proteins. Transmission of signaling via $G\alpha_i$ alone is sufficient for most GPCRs to initiate activation of both PLC and PI3K, and to promote degranulation of mast cells (17). FPRL1 and C3aR GPCRs link to $G\alpha_i$ in human mast cells as evidenced by their sensitivity to pertussis toxin and the ability of these receptors to degranulate human mast cells in our studies. Exceptions to this paradigm also exist. For example, $S1P_1$ receptor links to $G\alpha_i$ and induces mast cell chemotaxis but not degranulation. Whereas, $S1P_2$ receptor which, although linked to $G\alpha_i$, likely mediates its effects via $G\alpha_{12/13}$ or $G\alpha_q$ and degranulates mast cells (18). In accord, we showed that, although it did not degranulate human mast cells, C5L2 induced mast cell adhesion which was sensitive to pertussis toxin, suggesting that C5L2-induced adhesion may be mediated

via $G\alpha_i$. All of the three GPCRs, i.e. FPRL1, C3aR and C5L2, transduced signals through PI3K as demonstrated by the inhibition studies, further supporting with our hypothesis that chemotaxis and adhesion are G protein-dependent mechanisms in human mast cells, likely via $G\alpha_i$. The ability of specific GPCRs, such as prostaglandin receptor EP2 and β adrenergic receptor to down-regulate mast cell activation is dependent on the fact that these receptors stimulate adenylyl cyclase-dependent cyclic AMP (cAMP) production via $G\alpha_s$. cAMP has been demonstrated to negatively regulate mast cell function. The A_{2A} adenosine receptor links with $G\alpha_s$ and dampens FcεRI-mediated mast cell activation (18). Our studies revealed that A_{2A} receptor inhibited C3a-induced mediator release from human mast cells, likely via $G\alpha_s$, thus activating adenylyl cyclase. The A_{2A} receptor, however, was incapable of inducing degranulation on its own. In conclusion, both stimulatory and inhibitory G protein-dependent signal transduction pathway play roles in mast cell activation. While the stimulatory pathways are crucial for mast cell function in innate immunity and inflammation, the inhibitory pathways keep a check on unregulated mast cell activation in allergic reactions and hypersensitivity.

5.2 PERSPECTIVE

The variety of routes by which pathogen can activate mast cells during primary and secondary infection ensure that these cells have an effective sentinel role. Direct interaction with pathogens via TLR does not lead to degranulation (7, 19), although cytokine, chemokine and lipid mediator production have been reported. In our experiments, FPRL1 and C3a receptor activation led to degranulation of mast cells and the production of newly-generated mediators. Activation of C5L2 resulted in cytokine and chemokine production without degranulation. Based on our observations, we could postulate that during an infection these receptors respond to pathogens differentially in terms of time required to act and outcomes mediated at the site of infection. Following

the TLR, the C3aR is probably the first receptor system to respond to the complement fragment C3a generated early on during the complement activation cascade activated by PAMPs. This leads to rapid release of proinflammatory mediators required to combat infection as well as secretion of chemoattractants for recruitment of phagocytic leukocytes. Tissue resident mast cells located nearby recognize these chemoattractants and migrate towards the site of infection. It is when C5L2 system responds to infection facilitating mast cell migration without degranulation, thereby conserving cytokines and chemokines to be released later at the site of infection. Once mast cells have accumulated in high numbers, antimicrobial peptides released from the epithelial layer breached by pathogens bind the FPRL1 receptors and drive a robust mast cell response against infection.

Although all these systems have been investigated primarily as separate entities, an emerging body of evidence indicates extensive cross-talk between the N-formyl peptide, complement and TLR signaling pathways. Molecular interplay between these receptors can results in synergistic or even antagonistic interactions critical in reinforcing innate immunity or regulating excessive inflammation, respectively (20). Future studies should focus on defining such interactions *in vitro* as well as *in vivo* mast cell models. Future studies are also expected to elucidate additional regulatory links between stimulatory and inhibitory GPCRs and their cross-talk with PRRs, which is essential for understanding their precise roles in health and disease. The potential of mixing adenosine receptor agonists and antagonists with stimulatory GPCRs, such as C5aR and FPRL1, may lead to improved therapeutic interventions to enhance protective immunity or attenuate immunopathology.

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